The temperature- and growth phase-dependent regulation of the global virulence regulator RovA from *Yersinia pseudotuberculosis*

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Abbreviations

A	adenine
AHT	anhydrotetracycline
Amp	ampicillin
AP	alkaline phosphatase
ATP	adenosine triphosphate
BCIP	5-bromo-4-chloro-3-indolyl phosphate
bp	base pair
BSA	bovine serum albumin
C	cytosine
Cm	chloramphenicol
D	aspartic acid
Da	Dalton
DIC	differential interference contrast
DIG	digoxigenin
DNA	deoxyribonucleic acid
ds	double stranded
DTT	dithiothreitol
DYT	Double Yeast Tryptone
E	glutamic acid
EDTA	ethylenediaminetetraacetate
EMSA	Electro Mobility Shift Assay
G	guanine
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFP	green flourescent protein
hrs	hours
IPTG	isopropyl-β-thiogalactopyranoside
kb	kilobase
K_d	dissociation constant
kDa	kilo-Dalton
Kan	kanamycin
L	leucine
lacZ	reporter gene encoding β-galactosidase
LB	Luria-Bertani medium
M	molare
µg	microgram

mg	milligram
min	minute
µl	microlitre
ml	millilitre
mm	millimeter
mM	millimolar
N	asparagine
NBT	nitro blue tetrazolium
nm	nanometer
nt	nucleotide
OD	optical density
ONPG	o-nitrophenyl-β-galactoside
PAGE	polyacrylamide gelelectrophoresis
PCR	polymerase chain reaction
PFA	paraformaldehyde
phoA	reporter gene encoding alkaline phosphatase
PNPP	p-nitrophenyl phosphate
RNAP	RNA polymerase
rpm	revolutions per minute
Pr.	primer
RT	room temperature
SDS	sodium dodecyl sulfate
T	thymine
Taq	DNA-polymerase of *Thermus aquaticus*
Tet	tetracycline
T_m	melting temperature
Tris	tris-hydroxymethyl-aminomethane
T3SS	type 3 secretion system
V	valine
wt	wild-type

1 Introduction

Pathogenic bacteria need to adapt during their life-cycle to a variety of different and rapidly changing environmental conditions like temperature, pH, osmolarity, ion stress, oxygen stress and availability of nutrients. Therefore, gene expression needs to be tightly controlled to guarantee the survival inside and outside the host. Regulation of virulence factors either occurs on a transcriptional, post-transcriptional or post-translational level. Temperature and growth phase often play an important role to adjust the virulence program inside the host environment. The following chapters are supposed to give an overview of the complex regulation of *Yersinia* virulence gene expression.

1.1 The genus *Yersinia*

The genus *Yersinia* belongs to the family of *Enterobacteriaceae* and was named after the bacteriologist Alexandre Yersin (1863-1943) who first isolated the causative agent of the bubonic plague, *Yersinia pestis*. *Yersiniae* are rod-shaped, Gram-negative, facultative anaerobic bacteria that grow within a temperature range of 4°C to 43°C with an optimum of 25°C. Outside the host, most of the pathogenic *Yersinia* species are motile.

Among the 13 known *Yersinia* species only three are pathogenic to humans and animals: *Yersinia pestis*, *Yersinia enterocolitica* and *Yersinia pseudotuberculosis (Carniel, 2003)*. *Y. pestis* is highly virulent to humans and rodents (Bottone, 1997, Perry & Fetherston, 1997). Infection occurs via the bite of an infected flea and the injection of the bacteria into the bloodstream. Subsequently, *Y. pestis* proliferates extracellularly in the lymphatic tissues and leads to bubonic, septiceamic or pneumonic plague (Prentice & Rahalison, 2007).

The enteropathogenic species *Y. enterocolitica* and *Y. pseudotuberculosis* are taken up by ingestion of contaminated food or water (Bottone, 1997, Spinner *et al.*, 2008) and cause a variety of gut- and lymph-associated diseases (Yersiniosis). Infection of the gastrointestinal tract can lead to diarrhea, enterocolitis and mesenteric lymphadenitis but also to systemic infections and autoimmune diseases like reactive arthritis or erythema nodosum.

Although the epidemiology and disease pattern of *Y. pestis* differs strongly from the enteropathogenic species, the genome displays 97% homology to the genome of *Y. pseudotuberculosis* (Chain *et al.*, 2004) Therefore, it is assumed that *Y. pestis* diverged from *Y. pseudotuberculosis* 1.500 to 20.000 years ago (Chain *et al*., 2004, Wren, 2003, Achtman *et al.*, 1999). In addition to the 70 kb virulence plasmid pYV that is harbored by all three pathogenic *Yersinia* species, *Y. pestis* bears two additional virulence plasmids pFRA (100 kb) and pPLA (9.5 kb) which mediate the survival of the pathogen in the flea and the dissemination in the host's bloodstream, respectively (Cornelis, 1994, Chain *et al*., 2004). The genomes of *Y. pseudotuberculosis* and *Y. enterocolitica* show a much higher genetic diversity, indicating that both species already diverged around 41 to 186 million years ago (Achtman *et al*., 1999, Achtman *et al.*, 2004, Skurnik *et al.*, 2000).

Besides the different transmission routes and etiopathologies some general similarities between the different *Yersinia* species were found. All three human pathogens show a strong tropism to lymphatic tissues (Naktin & Beavis, 1999) and are able to efficiently counteract the host immune response by inhibition of macrophages and neutrophils as well as prevention of lysis by the complement system (Brandis, 1994, Straley *et al.*, 1993).

Due to their invading properties and the broad host spectrum enteropathogenic *Yersiniae* are excellent model organisms to study general infection mechanisms.

1.2 Infection route of *Yersinia pseudotuberculosis*

Enteropathogenic *Yersiniae* are taken up by contaminated food, especially pork and dairy products, and contaminated water (Naktin & Beavis, 1999, Dube, 2009, Bottone, 1997). In the early phase of infection they are able to survive in the acidic environment of the stomach and then reach and colonize the ileum. Peyer's patches, which are located within the small intestine, serve as entry ports for enteropathogenic *Yersiniae*. These organized lymphatic tissues are associated to the antimesenteric side of the small intestine and are covered by the follicle-associated epithelium (FAE) which contains M cells (microfold cells). This specialized cell-type samples antigens from the gut lumen and delivers it to antigen presenting cells (APCs), which are typically located basolaterally. By binding to β1-integrins, which are exclusively ex-

pressed on the surface of M cells, *Yersiniae* transmigrate and get inside the Peyer's patches (Isberg & Barnes, 2001).

Yersinia evolved strategies to counteract the first unspecific immune response after entry into the host cells by expressing antiphagocytic virulence factors (*Yersinia* outer proteins: Yops) (Viboud & Bliska, 2005, Trosky *et al.*, 2008). Evasion of the host's innate immune system allows extracellular bacterial replication and further spreading to the mesenteric lymph nodes (MLNs) and other organs like liver, spleen and kidney. In very few cases, mostly in immune compromised patients, *Yersinia*e disseminate into the bloodstream and cause a systemic infection (Marra & Isberg, 1996, Barnes *et al.*, 2006).

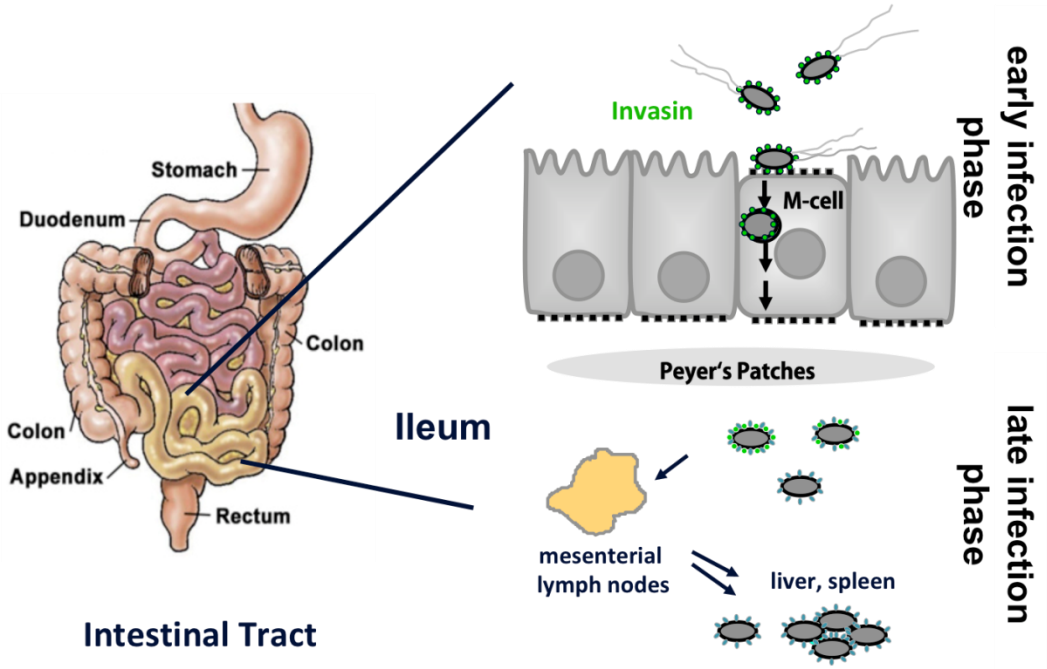


Figure 1-1. Infection route of *Yersinia pseudotuberculosis*. *Yersiniae* are taken up by contaminated food or water. In the early stage of infection they express the outer membrane protein invasin to mediate adhesion and invasion into M-cells in the epithelial layer of the ileum. After translocation, *Yersiniae* replicate in the underlying lymphatic tissue (Peyer's patches) and switch to expression of late virulence factors such as the adhesin YadA, the antiphagocytic Yops and the type III secretion system necessary to spread to the mesenteric lymph nodes and deeper tissues in the ongoing infection.

1.3 Virulence factors of *Yersinia pseudotuberculosis*

In order to effectively colonize their host, pathogenic *Yersiniae* need to synthesize virulence factors that enable them to attach and invade into the host tissue for replication and to counteract the immune response (Viboud & Bliska, 2005). These viru-

lence factors are either encoded on the chromosome or on the 70 kb virulence plasmid pYV. The virulence plasmid comprises the genes for the type III secretion system, the Yop effector proteins and the adhesion factor YadA. The invasion factor invasin is encoded on the chromosome of the pathogen.

1.3.1 Adhesion and invasion factors

Adhesion and invasion factors are expressed on the pathogen's surface and mediate the most crucial steps in host colonization. The expression of such factors takes place at different time points of infection. In the early stage of infection, *Yersiniae* need to express the invasion factor invasin, whereas in the later phase of infection, when the bacteria already reached the Peyer's patches, they start to express the second important virulence factor, the adhesin YadA. Besides those two major adhesion and invasion factors, other factors could be identified which share similar functions to invasin and YadA. The 17 kDa outer membrane protein Ail for example is encoded on the chromosome and mainly mediates adhesion and serum resistance (Bartra *et al.*, 2008, Kolodziejek *et al.*, 2007). Moreover, the pH6 antigen was shown to mediate hemagglutination and adhesion to mammalian cells (Yang & Isberg, 1997). Recently, additional invasin-like molecules of *Y. pseudotuberculosis* could be identified (F. Uliczka, unpublished data). The importance of these molecules in the infection process still needs to be investigated.

1.3.1.1 Invasin

The chromosomally encoded outer membrane protein invasin of *Y. pseudotuberculosis* mediates the invasion through the epithelial cell layer of the gastrointestinal tract into the underlying lymphatic tissues. The protein has a size of 103 kDa and its N-terminus is anchored to the bacterial outer membrane. The extracellular part of the protein consists of five β-stranded globular subdomains D_1-D_5. The subdomains D_4 and D_5 are located on the very C-terminus of the protein and are essential for efficient binding and uptake into eukaryotic cells (Leong *et al.*, 1991, Hamburger *et al.*, 1999, Dersch & Isberg, 2000). It was shown that the D_4-D_5 region is responsible for promoting cell attachment and entry by binding to β1-integrins on host cells (Clark *et al.*, 1998). The multimerization domain D_2 is located next to the N-terminal region of

the protein and mediates the formation of invasin multimers that bind to multiple β1-integrin receptors. This in turn leads to the clustering of integrins in the cell membrane, which enhances invasin-induced uptake by a zipper-like process (Dersch & Isberg, 2000, Isberg & Van Nhieu, 1995). The cell-to-cell contact triggers a signal cascade in the host cell that leads to rearrangement of the cytoskeleton. This results in the formation of pseudopodia which enclose the bacteria and internalize them into a membrane bound phagosome (Sansonetti, 2002).

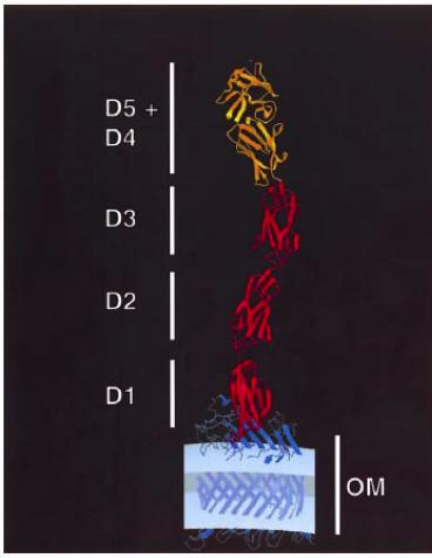


Figure 1-2. Crystal structure of the outer membrane protein invasin from *Y. pseudotuberculosis*. Invasin consists of five β-stranded subdomains (D_1-D_5) and is N-terminally anchored in the outer membrane. Domains D_4 and D_5 at the C-terminus mediate efficient binding and uptake through interaction with host cell integrins (Isberg *et al.*, 2000).

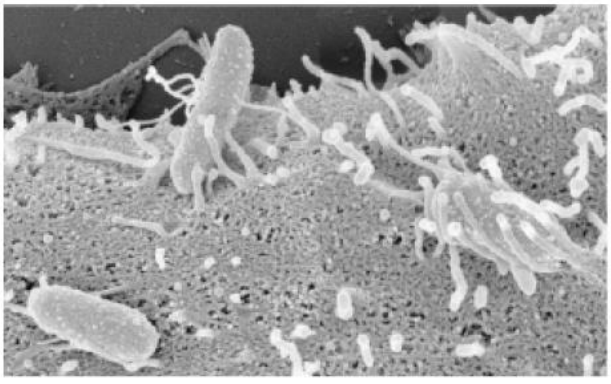


Figure 1-3. Electron microscopic picture of adhering *Y. pseudotuberculosis*, enclosed of host cell pseudopodia. Invasin mediated cell contact leads to activation of signal transduction cascades, which in turn results in the rearrangement of the host cell cytoskeleton. This induces the formation of pseudopodia and subsequently internalization of the pathogen into a membrane bound phagosome (Oezel, RKI, Berlin, 2005).

1.3.1.2 YadA

Yersinia adhesion factor A (YadA) mediates the adhesion and invasion into deeper tissues in the later phase of infection. The 432 amino acid protein is encoded on the virulence plasmid and belongs to the family of non-fimbrial Oca-adhesins (oligomeric coiled-coil adhesin). These proteins are outer membrane proteins with a trimeric lollipop-structure consisting of an N-terminal head domain, an intrinsic coiled-coiled formed stalk domain and a C-terminal membrane anchor.

YadA builds oligomers, consisting of 41 to 44 kDa monomers, leading to aggregates of 160 to 240 kDa that are exposed on the bacterial surface (Hoiczyk *et al.*, 2000, Koretke *et al.*, 2006). Binding to host cell integrins is mediated by a “bridging” mechanism via the extracellular matrix (ECM) proteins fibronectin, collagen and laminin (Eitel & Dersch, 2002, El Tahir & Skurnik, 2001).

In *Y. enterocolitica* the adhesin YadA lacks a 31 amino acid motif, which is characteristic for *Y. pseudotuberculosis* (YadA) and facilitates binding to β1-integrins via a fibronectin bridging mechanism (Heise & Dersch, 2006). Therefore, YadA from *Y. enterocolitica* is not able to bind fibronectin-bound β_1-integrin receptors but to collagen- and laminin-bound receptors. In contrast to both enteropathogenic *Yersinia* species, *yadA* is not expressed in *Y. pestis.* This is caused by a one base pair deletion at the beginning of the *yadA* coding region (El Tahir & Skurnik, 2001).

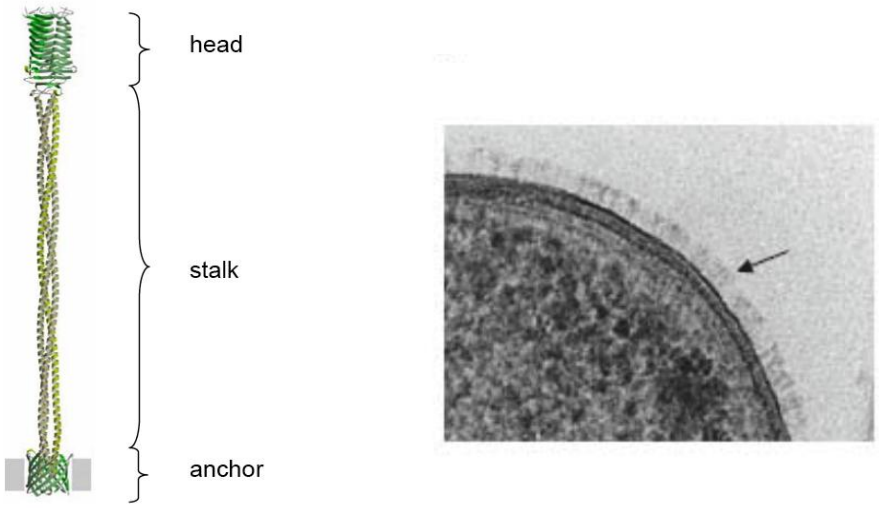


Figure 1-4. The outer membrane protein YadA. Crystal structure of *Y. enterocolitica* YadA (left side), showing head, stalk and anchor domains (Koretke *et al*., 2006) and electron microscopic picture of *Y. enterocolitica* expressing YadA (right side), showing the distribution of the lollipop-structured molecules on the bacterial membrane (Hoiczyk *et a*l., 2000).

The YadA head domain also mediates autoagglutination and hemagglutination (Skurnik *et al.*, 1984, Hoiczyk *et al*., 2000, Heise & Dersch, 2006). Furthermore, *Y. enterocolitica* YadA is able to block the host immune response by binding to complement inhibitor factors and is consequently circumventing the activation of the complement system (Balligand *et al.*, 1985, Kirjavainen *et al.*, 2008).

1.3.2 Type III secretion system and effector proteins

After transcytosis through the M cells the bacteria are immediately challenged by the host's innate immune system. Pathogenic *Yersinia* species developed an efficient strategy to counteract the attack by cells of the innate immune system. They require a type III secretion system to survive and replicate in the host lymphatic tissues. A set of pathogenicity factors, including those known as *Yersinia* outer proteins (Yops), is exported by this system upon bacterial infection (Heesemann *et al.*, 2006).

The genes of the proteins for the secretion apparatus (*ysc*) of the *Yersinia* type III secretion system and the Yops are encoded on the virulence plasmid pYV (Cornelis *et al.*, 1998). The *ysc*-genes are transcribed in the operons *virA*, *virB*, *virG* and *virC* (Cornelis *et al*., 1998). The Yop proteins are either secreted and translocated into the host cell as effector proteins or they are needed for efficient translocation of the other effector proteins (Viboud & Bliska, 2005, Heesemann *et al*., 2006, Matsumoto & Young, 2009). YopD and YopB together with LcrV form a pore in the eukaryotic cell (Cornelis, 2002a). Through this pore the effector proteins YopE, YopH, YpkA/YopO, YopM, YopJ/P and YopT are injected into the host and interfere biochemically with the target cell (Heesemann *et al*., 2006, Viboud & Bliska, 2005). YopE, YopH, YopO and YopT have been shown to counteract phagocytosis by macrophages and neutrophils (Viboud & Bliska, 2005). Furthermore YopJ/P is able to inhibit cytokine production and induce apoptosis (Orth, 2002, Ruckdeschel *et al.*, 2006, Trosky *et al*., 2008, Aepfelbacher, 2004). YopM was shown to interact with the cytoplasmic kinases RSK1 (ribosomal protein S6 kinase 1) and PKN2 (protein kinase C-like 2). How this interaction is relevant for Yersinia virulence is still unknown (McDonald *et al.*, 2003, Hentschke *et al.*, 2010).

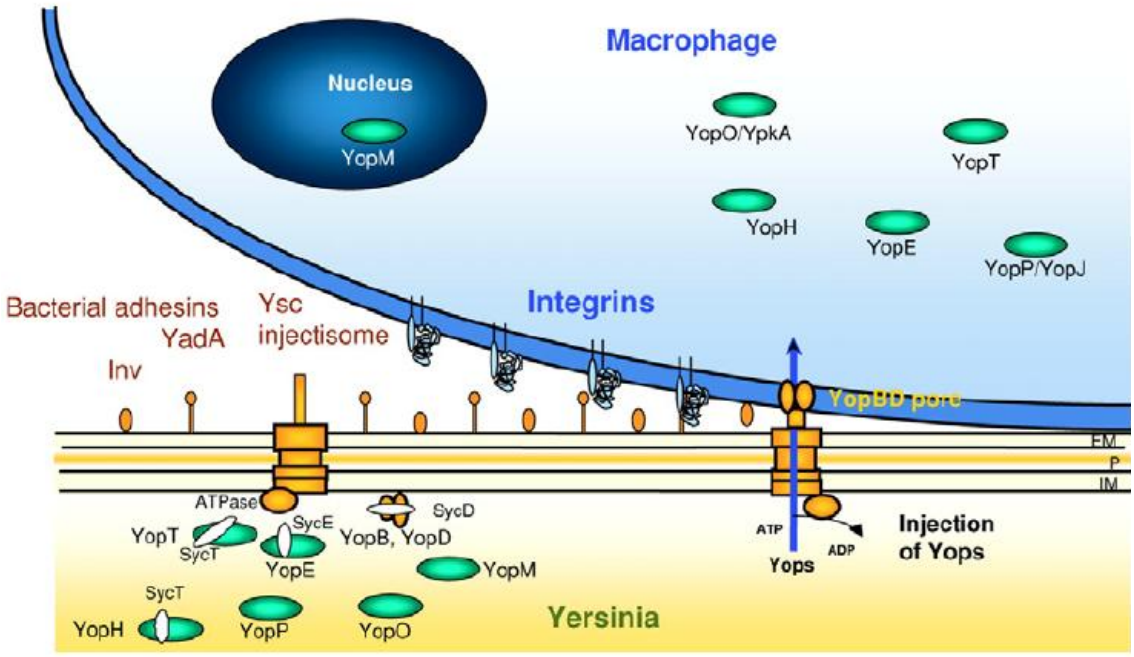


Figure 1-5. Secretion of Yops by the type III secretion system into host immune cells. After translocation through the M cells, *Yersinia* synthesizes the T3SS and the Yop proteins. Upon cell contact, mediated by YadA and invasin, Yops are injected into the host cell and interfere with the host's innate immune system (Cornelis, 2002b).

1.4 Environmental control of *Yersinia* virulence factors

Pathogenic bacteria need to be able to survive in the external environment as well as inside their hosts. Therefore, they are permanently exposed to changing environmental conditions and mechanisms are needed to ensure quick adaptation. The ability of sensing environmental parameters e.g. temperature, nutrient availability, osmolarity, ions or pH and subsequent reprogramming of virulence gene expression is an important feature for pathogenicity (Pepe *et al.*, 1994, Dube, 2009). The environmental control of *Yersinia* virulence gene expression has been well studied and is summarized in the next chapters.

1.4.1 Regulation of late virulence factors

After invasion and translocation through the M cells, the expression of late virulence factors is induced to ensure survival of *Yersiniae* and to allow them to colonize deeper tissues and organs. Many of those virulence factors are induced by higher temperatures (37°C). The Yop effector proteins and the Ysc injectisome that translocates

the Yops into the host cell, belong to the LCRS (Straley *et al*., 1993, Goguen *et al.*, 1984). The LCRS was shown to be repressed at 37°C in the presence of high amounts of Ca^{2+}, whereas induction was observed at 37°C under Ca^{2+} limiting conditions (Straley *et al*., 1993). This induction is mediated by the transcriptional activator LcrF (VirF in *Y. enterocolitica*), which is also strongly regulated by temperature on mRNA level (Hoe & Goguen, 1993). Furthermore, the YmoA protein seems to negatively influence *lcrF* transcription at moderate temperatures (K. Boehme, unpublished data).

The adhesin YadA, that is necessary for the colonization of deeper tissues, is maximally synthesized at 37°C in exponential growth phase in minimal medium. Similarly to Yop and Ysc, YadA synthesis is regulated by the transcriptional activator LcrF (Lambert de Rouvroit *et al.*, 1992). Eitel and Dersch (Eitel & Dersch, 2002) could show that addition of Mg^{2+} drastically increased *yadA* expression, whereas addition of Ca^{2+} had the opposite effect. Additionally, a cell contact dependent activation of *yadA* expression could be demonstrated (J. Eitel, S. Fehse and W. Opitz, unpublished data). At lower temperatures, YmoA indirectly represses *yadA* expression by inhibiting LcrF synthesis (Cornelis *et al.*, 1991, K. Boehme, unpublished data).

1.4.1.1 Regulation of early virulence factors

The early virulence factors are only needed for the initial steps of infection, the invasion into the host cells. Expression in later infection stages would increase the risk of being recognized by the immune system. Surprisingly, some of the early infection phase factors are also produced at moderate temperatures, at conditions resembling the environment outside the host. Besides the most important early phase virulence factor invasin, flagella, the LPS, iron uptake systems and toxins can be included into the group of early virulence factors.

Flagella are only synthesized at moderate temperatures, and seem to facilitate penetration of the epithelial cells after colonization of the ileum (Badger & Miller, 1998). The LPS has an altered structure at moderate temperatures. The O-antigen, that plays a role in effective colonization of host tissue and serum resistance, was demonstrated to be down regulated at 37°C in *Y. enterocolitica* and *Y. pseudotuberculosis* (Skurnik, 2003).

Toxins of enteropathogens are able to cause severe damages in the intestinal tract of the host. *Y. enterocolitica* but not *Y. pseudotuberculosis* secretes the heat-stable enterotoxin Yst, which is highly similar to STI of *E. coli*. Yst is maximally synthesized at 25°C in stationary growth phase and seems to be important for persistence and dissemination of the pathogen (Delor & Cornelis, 1992).

Upon entering the host cell, iron becomes the limiting factor for bacterial growth. For efficient replication and infection, they need to take up the iron from the host environment. Therefore, bacteria developed high affinity iron scavengers called siderophores. In *Yersinia* iron uptake is mediated by the Yersiniabactin system, which is expressed in the early phase of infection and is positively regulated by the AraC-like transcriptional regulator YbtA and negatively influenced by the ferric uptake regulator Fur (Carniel *et al.*, 1996, Carniel, 2001).

The outer membrane protein invasin is the most important virulence factor in the early phase of infection. Expression of the *inv* gene was found to be regulated by environmental signals such as temperature, growth phase, nutrient availability and pH (Pepe *et al*., 1994, Revell & Miller, 2000, Nagel *et al.*, 2001). Maximal *inv* expression is observed in stationary growth phase at 25°C at neutral pH, whereas low expression was found at 37°C in exponential growth phase in *Y. enterocolitica* and *Y. pseudotuberculosis* (Revell & Miller, 2000, Nagel *et al*., 2001). Furthermore, *inv* expression in *Y. pseudotuberculosis* is induced in complex media at low osmolarity, but reduced in minimal media (Nagel *et al*., 2001).

The global transcription factor RovA was identified to regulate the environmental controlled *inv* expression in *Y. enterocolitica* and *Y. pseudotuberculosis* (Nagel *et al*., 2001, Revell & Miller, 2000).

1.5 The global virulence regulator RovA

The regulator of virulence A (RovA) is a 143 amino acid protein that belongs to the dimeric winged-helix family of SlyA/Hor transcription regulators found in many *Enterobacteriaceae*. These regulators play an important role in the environmental adaptation, virulence and survival inside the host and are able to positively or negatively influence gene expression (Ellison & Miller, 2006). RovA was first discovered as a regulator of *inv* expression by transposon mutagenesis in *Y. enterocolitica* (Revell &

Miller, 2000) and shortly after in *Y. pseudotuberculosis* by complementing the induction of an *inv-phoA* fusion in *E. coli* (Nagel *et al*., 2001).
The protein directly interacts with two separate regions between position -207 and -50 in the *inv* regulatory region and induces activation of *inv* expression (Nagel *et al*., 2001).
In *E. coli*, the RovA homolog SlyA is responsible for the induction of the cryptic hemolysin ClyA and regulates a large variety of proteins, involved in metabolism, transport, acid resistance and starvation (Spory *et al.*, 2002). SlyA in *Salmonella* is important for stress resistance, survival in macrophages and for virulence (Buchmeier *et al.*, 1997, Daniels *et al.*, 1996, Stapleton *et al.*, 2002, Navarre *et al.*, 2005). Furthermore, SlyA regulates the pathogenicity island SPI2 and SPI2-associated genes in *S. typhimurium* (Buchmeier *et al*., 1997, Linehan *et al.*, 2005) and is responsible for the repression of its own gene (Norte *et al.*, 2003, Shi *et al.*, 2004). *E. coli* SlyA is involved in the regulation of capsule transcription in an H-NS dependent manner (Corbett *et al.*, 2007). Expression of *slyA* is positively autoregulated and dependent on temperature and growth phase. Maximal *slyA* expression is observed at 37°C in exponential growth phase (Corbett *et al*., 2007). In contrast, SlyA of *S. typhimurium* is negatively autoregulated and maximally expressed in stationary growth phase (Stapleton *et al*., 2002, Buchmeier *et al*., 1997). Recently, an indirect regulatory role for the alternative sigma factor RpoS on *slyA* expression in *E. coli* could be demonstrated (H. Langhans, unpublished data). Other RovA homologues like PecS/Hor/SlyA from *Erwinia/Dickeya* species and Rap in *Serratia marcescens* regulate the synthesis of secondary metabolites such as pigments and antibiotics as well as cellulases and pectate lyases, involved in plant pathogenicity (Thomson *et al.*, 1997, Haque *et al.*, 2009).

1.5.1 Influence of RovA on *Yersinia* physiology and virulence

Similarly to other members of the SlyA/Hor family, RovA was shown to be a global transcriptional regulator that influences the expression of multiple genes involved in virulence, physiology, stress adaptation and metabolism.
Mouse infection studies have shown that *rovA* mutant strains of *Y. enterocolitica* and *Y. pseudotuberculosis* exhibit an increased LD_{50} compared to the wild-type and the *inv* mutant strain. Furthermore it could be shown that the *rovA* mutant strains have a

reduced ability to colonize the Peyer's patches and to disseminate into deeper tissues (Revell & Miller, 2000, Heroven & Dersch, 2006, Nagel *et al.*, 2003). In addition, a *rovA* mutant but not an *inv* mutant did not induce the expression of IL-1α and the characteristic inflammatory response in the Peyer's patches (Dube *et al.*, 2001).
Y. pestis is lacking a functional *inv* gene, but interestingly, the RovA protein is still required for virulence. *rovA* mutants of *Y. pestis* are drastically less virulent after subcutaneous inoculation (Cathelyn *et al.*, 2006). Furthermore, survival experiments demonstrated that a *Y. pseudotuberculosis rovA* mutant is hypersensitive to hydrogen peroxide and high concentrations of salt (Nagel *et al*., 2003). Taken together, these results indicate that RovA is not only a regulator of *inv* synthesis but seems to be a global transcriptional regulator. 2D gel electrophoresis revealed that at least 50 genes are up- or down-regulated in a *Y. pseudotuberculosis rovA* mutant compared to the wild-type (Nagel *et al*., 2003), Moreover, a set of 32 different promoters, directly interacting with the *Y. pseudotuberculosis* RovA protein, was discovered (H. Tran-Winkler, unpublished). This set includes promoter regions of genes involved in metabolism and stress adaptation, but also nucleoside transporters, iron sequestration systems and adhesins like the pH6 antigen. These results could be confirmed by microarray analysis and led to the identification of further RovA regulated genes, mostly involved in *Yersinia* metabolic pathways (AK. Heroven, unpublished data).

1.5.2 Structure and function of RovA

The members of the SlyA/Hor family share a low similarity ($\leq$ 40%) to the MarR- (multiple antibiotic resistance regulator) family of transcriptional regulators, that are widely distributed among many bacterial and archaeal species and are involved in the regulation of diverse physiological processes. Members of this family are for example the repressor proteins MarR, PecS, MprA, MexR, OhrR, HucR and $SlyA_{EF}$.
MarR, the most prominent regulator of this family, is a repressor of the *marROAB* operon, mediating multiple antibiotic resistances in *E. coli* (Alekshun & Levy, 1999a, Martin & Rosner, 1995). The MarR crystal structure revealed that the protein is a homodimer, with each monomer consisting of six α-helices and two β-sheets. DNA-binding is mediated by a winged helix-turn-helix motif in each monomer that includes the α-helices α_3 and α_4 as well as the two following β-sheets. The terminal regions of each monomer were postulated to be necessary for dimerization (Alekshun *et al.*,

2000, Alekshun *et al.*, 2001). All MarR homologues bind sequence specific to palindromic or pseudopalindromic sites on the DNA. This binding often involves a specific anionic lipophilic ligand (Wilkinson & Grove, 2006).

Functional analysis of RovA revealed a strong homology to the MarR type regulators. Cross-linking and gel filtration experiments showed that RovA forms stable homodimers and dimerization seems to be mediated by the first N-terminal and the last two C-terminal helices (Tran *et al.*, 2005). Furthermore, comparison of the RovA sequence with other MarR-type regulators revealed a strong homology of the DNA-binding domain that consists of a helix-turn-helix motif, followed by two β-sheets (wing). Mutational analysis and DNA-binding studies proved this motif to be directly involved in DNA-binding (Tran *et al*., 2005), presumably by interacting with the major groove of the DNA. Additionally, the last four amino acids of the C-terminus were shown to be crucial for activation of gene transcription by direct interaction with the α-CTD of the RNA-polymerase, allowing RovA to specifically activate virulence gene expression (Tran *et al*., 2005).

In order to identify the RovA binding sites DNA-binding studies were first performed with the *inv* promoter region. It could be shown that RovA binds to two separate, non-palindromic sequences, both necessary for full activation of gene expression (Heroven *et al.*, 2004). Introduction of modifications into this sequences revealed that the sequence $^{A/T}$ATTAT$^{A/T}$ is important for RovA-DNA interaction.

Recently, the RovA crystal structure was solved, confirming both, the structural model and the predicted DNA-binding sequence. Additionally, it was shown that RovA is able to unspecificly bind to DNA via interactions with the phosphate residues in the DNA-backbone (N. Quade, unpublished data).

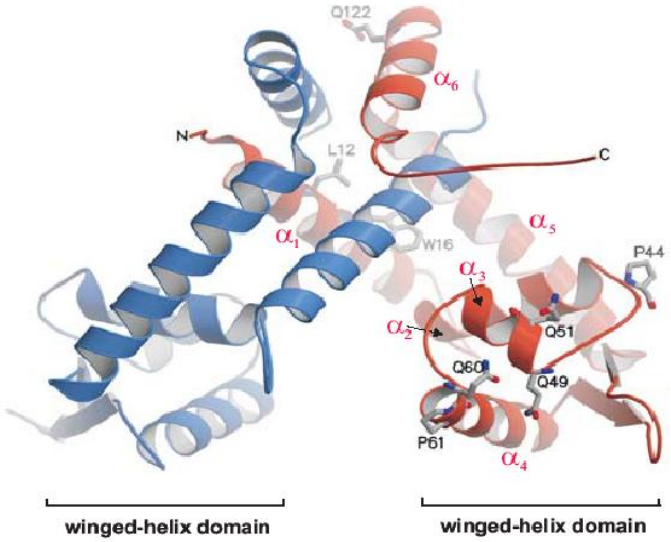


Figure 1-6. Structural model of the global virulence regulator RovA. The two monomers are colored in blue and red. The N- and C-terminus are indicated as well as the six α-helices and the substituted functional residues, leading to loss of RovA function. Each monomer possesses a winged helix-turn-helix motif involved in DNA-binding. Dimerization is mediated by the helices α_1, α_5 and α_6, whereas the four last C-terminal amino acids interact directly with the RNA polymerase and activate transcription (Tran *et a*l., 2005).

1.5.3 Regulation of *rovA* expression

rovA expression is strictly controlled by environmental parameters, e.g. temperature, growth phase, availability of nutrients, osmolarity and pH. Maximal expression is observed at moderate temperatures in stationary growth phase when high amounts of nutrients are available. In contrast, only very low amounts of the protein are found at 37°C in exponential growth phase (Nagel *et al*., 2001).

1.5.3.1 Autoregulation of RovA

RovA was found to be transcribed by two different promoters P1 and P2 mapped 76 and 343 nt upstream of the translational start site (Heroven *et al*., 2004). Sequences upstream and downstream of these promoters interact specifically with the RovA protein and are required for a concentration-dependent autoregulatory mechanism. The first binding region within the *rovA* promoter is located at position -526 to -422 and is composed of several binding sites with high affinity to RovA. The second binding region is located downstream of P1 between position +44 to +68 and exhibits low bind-

ing affinity to RovA. Binding to this second site was observed at high intracellular RovA levels and reduces expression of the *rovA* gene. Therefore, binding site II was described as a threshold valve that is occupied when a certain RovA level in the cell has been reached (Heroven *et al*., 2004).

Besides the positive and negative autoregulation, *rovA* expression was found to be mediated by a variety of regulatory factors that form a complex regulatory network and directly (H-NS and RovM) or indirectly (Csr-system, YmoA) interact with the *rovA* promoter region, activating or repressing *rovA* expression (Heroven *et al.*, 2004, Heroven & Dersch, 2006, Heroven *et al.*, 2008, Heroven, 2010). In the following chapters these factors are described in detail. A summary of the whole RovA regulatory network is presented in Figure 1-7.

1.5.3.2 The nucleoid-associated protein H-NS

H-NS is known to play a major role in temperature-dependent regulation of virulence and stress resistance genes in various Gram-negative enteropathogenic bacteria (Ono *et al.*, 2005, Schroder & Wagner, 2002, Atlung & Ingmer, 1997). The small dimeric protein consists of a C-terminal DNA-binding domain and an N-terminal oligomerization domain, both connected by a flexible peptide linker (Dorman *et al.*, 1999, Badaut *et al.*, 2002). It binds preferentially to intrinsically curved DNA with AT-rich sequences that are predominantly found in promoter regions (Atlung & Ingmer, 1997, Dorman, 2004). Sequences harboring the motif 5'-TCG ATA TAT T-3' were found to be bound with higher affinity than other AT-rich motives. Usually multiple H-NS proteins bind and oligomerize at these AT-rich regions along one DNA molecule (loop formation) or two separate DNA-molecules (bridging), preventing binding of the RNAP and thus, silencing gene transcription (Williams & Rimsky, 1997, Rimsky *et al.*, 2001).

Virulence genes are generally expressed at 37°C but silenced by H-NS at temperatures below 30°C (Rimsky *et al*., 2001, Badaut *et al*., 2002). In *Y. pseudotuberculosis* an opposite mechanism was found: at 37°C H-NS binds to AT-rich sequences between position -201 and -31 in the *inv* regulatory region and between positions -547 and -429 in the *rovA* promoter region. These H-NS binding regions overlap with the RovA binding regions in both promoter regions. At moderate temperatures RovA is synthesized and exhibits a very high affinity for the binding-sites within these promo-

ter regions, thereby counteracting repression by H-NS (Heroven *et al*., 2004). A similar repressing effect was shown for *inv* transcription in *Y. enterocolitica* (Ellison & Miller, 2006).

1.5.3.3 The LysR-type regulator RovM

Genetic screening for components of the *rovA* regulatory cascade led to the identification of the LysR-type regulator RovM (modulator of *rovA* expression). LysR-proteins are co-inducer-responsive oligomeric proteins with a helix-turn-helix DNA-binding motif at the N-terminus, a C-terminal inducer binding regulatory domain and a flexible linker-helix involved in oligomerization (Schell, 1993). Recently, the structure of the RovM inducer-domain has been solved, proposing RovM to be a tetrameric molecule with two distant DNA-binding domains and a cavity for binding an inducer molecule (Quade *et al*., 2010). Sequence alignments with other LysR-regulators revealed high similarity to the DNA binding domains of CynR from *E. coli*, CbnR from *Ralstonia eutropha* and BenM from *Acinetobacter* sp., but low identity of the regulator domain, indicating a conserved binding mechanism but different inducer molecules (Quade *et al.*, 2010).

RovM from *Y. pseudotuberculosis* binds to a short palindromic region close to the upstream promoter P1 (-80 to -47), thereby repressing *rovA* transcription in cooperation with the histone-like protein H-NS. The *rovM* gene itself is positively autoregulated but no specific RovM binding site in the *rovM* promoter region was found (Heroven & Dersch, 2006). Therefore, indirect autoregulation of RovM cannot be excluded. *rovM* expression is significantly induced during growth in minimal medium but is not affected by temperature or growth phase, suggesting that the regulation of *rovA* expression in response to nutrient availability is mediated through RovM (Heroven & Dersch, 2006). Deletion of the *rovM* gene leads to increased RovA and invasin synthesis and enhances internalization of *Yersinia* into host cells. Furthermore, a *rovM* mutant was shown to be more virulent and more invasive than the wild-type in the mouse infection model (Heroven & Dersch, 2006). Consistent with this, overexpression of RovM results in attenuated dissemination of *Y. pseudotuberculosis* into deeper tissues. Moreover, RovM overexpression results in increased motility of the pathogen (Heroven & Dersch, 2006). In *Y. pseudotuberculosis, rovM* expression in

response to the nutrient content is regulated by the carbon storage regulatory system (Csr) (Heroven *et al*., 2008).

1.5.3.4 The Carbon storage regulatory (Csr) system

Besides H-NS and RovM, genetic screenings to identify components influencing *rovA* expression revealed factors with homologies to components of the Carbon storage regulatory system (Heroven & Dersch, 2010). The Csr-system in *Y. pseudotuberculosis* consists of the small regulatory RNAs CsrB and CsrC and the RNA-binding protein CsrA and was found to affect *rovA* expression by regulating synthesis of RovM *(Heroven et al*., 2008). In contrast to other regulatory RNAs that directly interact with the mRNA of a target gene, CsrC and CsrB are able to bind to the mRNA-regulating protein CsrA. This binding is mediated by a typical motif (5'-GAA/RGGA-3') in the loop regions of the RNAs and leads to sequestration of CsrA and thus, to inactivation of the protein (Gutierrez *et al.*, 2005, Mercante *et al.*, 2009).

Overexpression of CsrC and CsrB leads to increased *rovA* transcription whereas overexpression of the CsrA protein mediates the opposite effect. In fact, when a *csrA* mutant is grown in minimal medium, RovA is produced in high amounts. Under these conditions, *rovA* expression is completely abolished by RovM repression. Therefore it can be concluded that repression of *rovA* in a *csrA* mutant occurs via down regulation of *rovM* (Heroven *et al*., 2008). *csrC* expression is strongly repressed in minimal media but maximally induced in complex media, indicating that nutrient rich growth conditions may lead to sequestration of CsrA by CsrC, which consequently results in decreased levels of RovM followed by higher synthesis of RovA. Furthermore, *csrC* expression was shown to be induced by the histone-like protein YmoA (K. Boehme, unpublished data). An inducing effect on CsrC was also found by the RNA-binding protein Hfq and the cAMP receptor protein Crp (AK. Heroven, unpublished data). Moreover, Fe^{3+} seems to influence *csrC* expression (S. Nordlohne, unpublished data), implying iron-responsiveness of the Csr-system. Expression of *csrB* was very low under all tested media conditions and was found to be positively regulated by the two-component system BarA/UvrY, leading to repression of *csrC* via a yet unknown mechanism (Heroven *et al*., 2008). Moreover, CsrB seems to be negatively influenced by the cAMP receptor protein Crp (AK. Heroven, unpublished data).

Additionally the Csr-system affects growth, morphology, carbon metabolism, stress resistance, motility and entry into epithelial cells (AK. Heroven, unpublished data). Thus, a tight control of expression and activity of the Csr-system components is crucial for optimal expression of virulence genes during infection.

1.5.3.5 The histone-like protein YmoA

The nucleoid-associated protein YmoA belongs to the family of Hha/YmoA proteins, a group of low molecular-mass proteins, functionally equivalent to the N-terminus of H-NS, that are involved in (virulence) gene regulation in a variety of Gram-negative bacteria (Stoebel *et al.*, 2008). YmoA was shown to interact with H-NS or H-NS homologous proteins, leading to the formation of heterodimeric complexes (Nieto *et al.*, 2002, Madrid *et al.*, 2007)

In *Y. pseudotuberculosis* YmoA is involved in the regulation of several virulence factors that are encoded on the virulence plasmid pYV, i.e. YadA, the Yop effector proteins and components of the T3SS (Lambert de Rouvroit *et al*., 1992, Cornelis *et al*., 1991). These virulence genes are activated by the AraC-type regulatory protein LcrF at 37°C (Bolin *et al.*, 1988) and are repressed by YmoA at moderate temperatures (Cornelis *et a*l., 1991). YmoA of *Y. pestis* is known to be degraded by the Lon and ClpXP proteases at 37°C in exponential growth phase (Jackson *et al.*, 2004). The same effect could be shown for YmoA of *Y. pseudotuberculosis* (K. Boehme, unpublished data).

Furthermore, it was shown that *rovA* expression was drastically reduced in an *ymoA*-mutant suggesting a role for YmoA in *rovA* activation and *inv* expression. Such activation occurs indirectly via RovM and the Csr-system: YmoA, most likely in cooperation with the histone-like protein H-NS, induces CsrC synthesis, which, in turn, sequesters CsrA and therefore represses RovM synthesis. This process seems to be media dependent (K. Boehme, unpublished data). Thus, it is postulated that YmoA plays a crucial role in switching from RovA-activated expression of early virulence genes to LcrF-mediated late virulence gene expression.

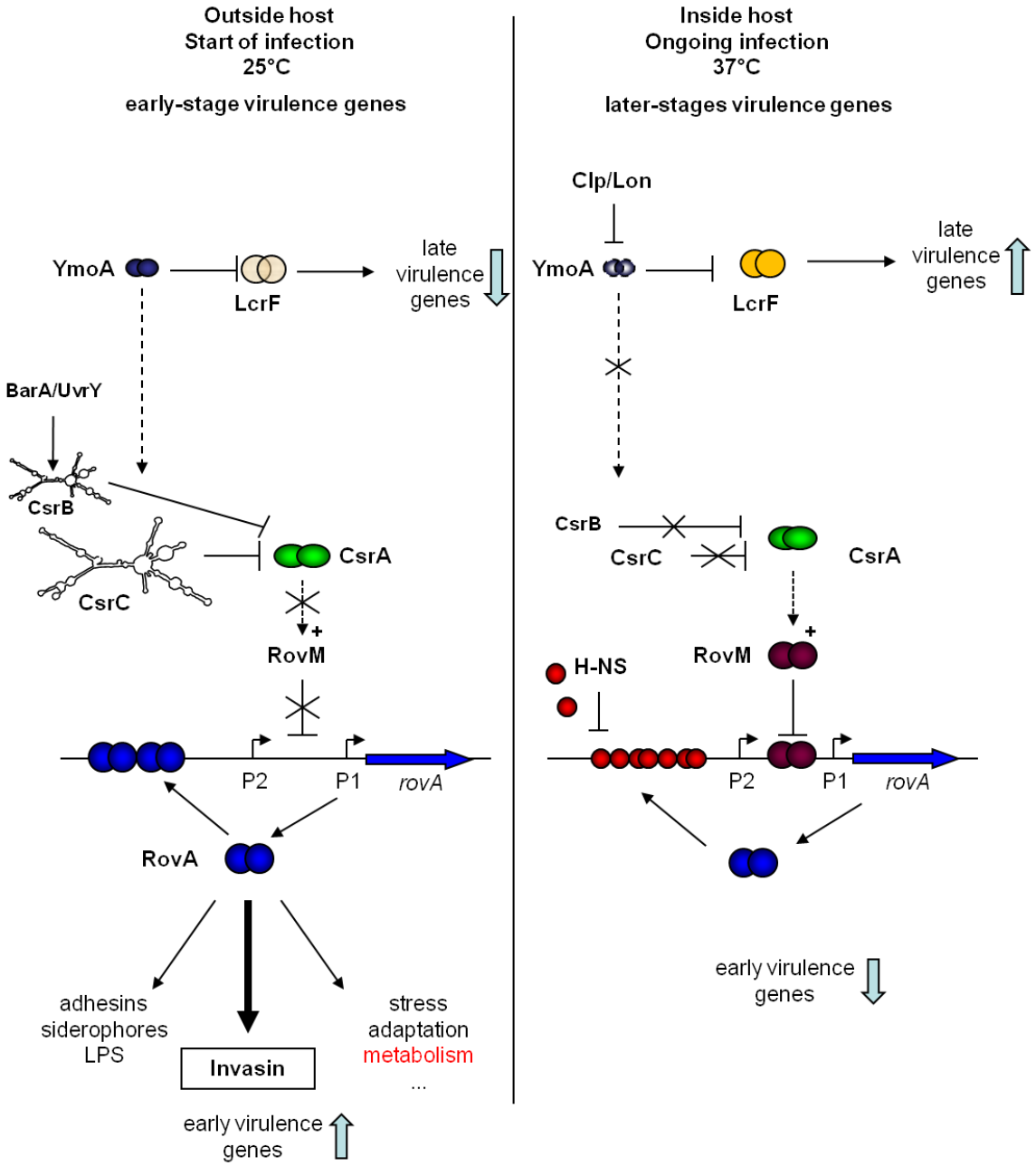


Figure 1-7. The RovA regulatory network. At moderate temperatures, *Y. pseudotuberculosis* synthesizes the virulence regulator YmoA that represses LcrF, the key regulatory protein for plasmid encoded late virulence genes, and induces synthesis of the small regulatory RNA CsrC. CsrC and CsrB sequester CsrA that is not able to stabilize the *rovM* transcript. RovA activates its own transcription, leading to expression of early virulence genes and genes involved in stress adaptation and metabolism. During the ongoing infection YmoA is degraded by the Lon and Clp proteases, LcrF is transcribed and leads to the expression of late virulence genes. CsrC synthesis is repressed, enabling CsrA to activate RovM expression. RovM represses *rovA* transcription together with the nucleoid-associated protein H-NS, leading to inactivation of early virulence gene expression.

1.6 Mechanisms of temperature and growth phase dependent gene regulation

During their life-cycle bacteria are exposed to a variety of different and rapidly changing environmental conditions such as temperature, pH, osmolarity and availability of oxygen and nutrients. Especially for pathogenic bacteria temperature is one of the most important signals to change their gene expression program upon entry into the host organism (Konkel & Tilly, 2000). Temperature changes can either be sensed in an indirect way e.g. by accumulation of denatured proteins (heat shock) or stalled ribosomes (cold shock) or in a direct way by so called thermosensors. So far, three types of thermosensors have been described controlling gene expression on a transcriptional, translational or post-translational level (Hurme & Rhen, 1998, Schumann, 2009, Klinkert & Narberhaus, 2009).

One of the most important steps in transcriptional gene regulation is the binding of the RNA polymerase to the promoter region and the formation of the initiation complex (Gourse *et al.*, 2006). The presence of intrinsically curved DNA regions strongly influences the affinity of the RNA polymerase to the promoter. Since DNA curvature and supercoiling are affected by temperature, any temperature-induced change of DNA-topology will consequently have an impact on gene expression (Klinkert & Narberhaus, 2009). A direct effect of changes in DNA-topology was for example shown for the promoter region of the phospholipase C (*plc*) in *Clostridium perfringens* (Katayama *et al.*, 1999) or for the omega-3 desaturase gene (*desB*) in *Synechocystis* (Los, 2004).

Binding to specific silencer proteins which modulate DNA-topology may lead to temperature-induced repression of transcription in an indirect way. One of those silencer proteins is the nucleoid-associated protein H-NS that is involved in the temperature-dependent regulation of the *Shigella flexneri* virulence regulator VirF (Falconi *et al.*, 1998, Prosseda *et al.*, 1998). At temperatures below 32°C H-NS binds to two different binding sites on the *virF* promoter region separated by a curve in the DNA structure. The curved DNA directly serves as a thermosensor. With increasing temperature the conformation of the *virF* promoter changes, leading to decrease in H-NS repression and subsequently, transcription of *virF (*Prosseda *et al., 2004,* Falconi *et al.*, 1998).

The transcriptional control of temperature-induced gene expression is mediated by RNA thermometers. These thermometers respond to temperature by melting of the

RNA structure. At low temperatures the Shine-Dalgarno sequence and the start codon in the 5'-UTR of the mRNA form a loop structure and prevent binding of the ribosome. At higher temperatures the structure melts and allows the initiation of transcription. In *Listeria monocytogenes* for example, an RNA thermometer was found to control the regulation of the virulence gene regulator PfrA (Johansson *et al.*, 2002). Folding of the *prfA* mRNA prevents ribosome binding at lower temperatures. At 37°C the structure is destabilized, enabling translation of *prfA* when the pathogen enters the mammalian host (Johansson *et al.*, 2002).
Thermosensing may also occur on a post-translational level. The tertiary and quaternary structures of proteins are very susceptible to changes in temperature. This can lead to conformational changes, thus activating or inactivating the protein. Moreover, the disassembly of a dimer into a monomer could be observed (Klinkert & Narberhaus, 2009).
A temperature-sensing ability was demonstrated for the transcriptional regulators TlpA of *Salmonella typhimurium* (Hurme *et al.*, 1997) and RheA of *Streptomyces albus* (Servant *et al.*, 2000). Both proteins are able to reversibly change their conformation upon temperature shifting. This leads to altered DNA-binding abilities, directly influencing gene transcription.

Besides the changes of temperature, bacteria are also confronted with changes in nutrient concentration upon entering the host organism. For enteropathogenic *Yersiniae* for example, nutrient availability outside the host environment is generally low, resembling stationary phase conditions, whereas conditions inside the gut are more comparable to those in exponential growth phase.
Growth phase dependent gene expression was found to be regulated by a variety of cellular processes and components, including DNA-supercoiling, the alternative sigma factors RpoS, quorum sensing molecules and the stringent response (Navarro Llorens *et al.*, 2010, Lazazzera, 2000).
As described previously, the DNA- topology is important for transcriptional regulation of bacterial gene expression. The degree of supercoiling varies not only with temperature but also with growth conditions (Dorman, 1996) and is controlled by several regulatory pathways including sigma factors, ppGpp and the nucleoid-associated proteins H-NS, Fis and Crp (Travers & Muskhelishvili, 2005).

Entry from exponential into stationary phase results in induction of the alternative sigma factor σ^S (RpoS). Consequently, transcription of numerous RpoS-dependent genes is activated, many of which encode gene products with stress-protective functions such as chaperones and proteases (Hengge-Aronis, 2002). Regulation of RpoS occurs at the levels of translation, transcription, proteolysis and protein activity (Hengge, 2009). In *Salmonella* most of the genes of the starvation-stress response (SSR) stimulon are under positive control of RpoS. This stimulon includes transporter systems, metabolic enzymes, regulatory proteins and virulence factors (Spector, 1998). The plasmid-encoded virulence regulon *spvABCD* for example is expressed under stationary phase conditions and was demonstrated to be controlled by RpoS (Guiney *et al.*, 1995). Moreover, a *Salmonella rpoS* mutant was significantly reduced in virulence of mice. In contrast, the involvement of RpoS in virulence gene expression in *Y. enterocolitica* could not been shown (Badger & Miller, 1995).

Furthermore, several bacteria were found to integrate starvation and quorum sensing signals to regulate the transition into stationary growth phase (Lazazzera, 2000). In *Pseudomonas aeruginosa* for example, the transcription of the gene encoding RpoS is positively regulated by homoserine lactones (You *et al.*, 1998, Latifi *et al.*, 1996). Moreover, quorum sensing can be activated by the stringent response (van Delden *et al.*, 2001).

The stringent response is a stress response mechanism induced by amino acid starvation and is mediated by the accumulation of ppGpp (Navarro Llorens *et a*l., 2010). ppGpp concentrations are adjusted by the ppGpp synthase RelA and by the ppGpp synthase/hydrolase SpoT (Gentry *et al.*, 1993). ppGpp binds to the β-subunit of the RNA polymerase, affecting promoter specificity. Moreover, *rpoS* expression is positively regulated by ppGpp (Gentry *et al.*, 1993). When nutrient supplies increase, the ppGpp levels decrease and the stringent response is reversed.

1.7 Aim of this study

In *Yersinia pseudotuberculosis* the expression of early virulence genes is regulated by the global transcriptional regulator RovA which itself is regulated via a complex regulatory network. RovA is maximally expressed at 25°C during stationary growth phase, whereas minimal expression was observed at 37°C in the exponential growth phase. Although various regulators of RovA have been identified within the regulatory

network none of them was found to be responsible for a direct temperature and growth phase dependent expression of *rovA*. Previous work on RovA regulation indicated a post-translational mechanism. Further investigation of this mechanism indicated that RovA might be able to directly respond to temperature changes. Moreover it was hypothesized, that temperature induced conformational changes influence the susceptibility of RovA to the Lon and Clp proteases (M. Bujara, unpublished data).
This work aims at validating these hypotheses by closer investigation of temperature- and growth phase-dependent regulation of RovA.

2 Materials and Methods

2.1 Materials

Chemicals, media and consumables were purchased from Sigma-Aldrich, Merck, Roth, VWR, Roche, Ibidi, Millipore, Fermentas, Finnzyme, Promega, BioRad, Schleicher & Schuell, Biochrom AG, BD, GIBCO, AppliChem, Roche, and Oxoid.
Restriction enzymes, ligases, polymerases and other enzymes used in this study were obtained from New England Biolabs, Finnzyme, Merck, Promega or Agilent Technologies. Kits for DNA preparation, purification and gel extraction were purchased from Qiagen, DNA and protein standards from Fermentas.

2.2 Bacterial strains and growth conditions

E. coli and *Yersinia pseudotuberculosis* strains used in this study were cultivated in liquid media at 200 rpm on incubation shakers (Infors Multitron) or on agar plates at 37°C or 25°C and are listed in Table 1. If not indicated otherwise bacteria were grown on LB medium (Luria-Bertani) or on LB agar plates (Sambrook *et al*., 1989). For certain experiments bacteria were grown in DMEM/F12 (Biochrom), M9 (Sambrook *et al*. 1989) or MMA (Sambrook *et al*. 1989). Minimal media were supplemented with 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.2% casamino acids, 0.2% glucose and 1 µg/ml thiamine after sterilization. Antibiotics were supplied in the following concentrations: carbenicillin 100 µg/ml, chloramphenicol 30 µg/ml, kanamycin 50 µg/ml and tetracycline 5 µg/ml.
For promoter induction AHT (anhydrotetracycline) (0.2 mg/ml), arabinose (0.2%) or IPTG (Isoporopyl-β-D-thiogalactopyranoside, 100 µM) was used. Cultures for preparation of competent cells and for phenotypic expression of transformed cells were grown in DYT (Double Yeast Tryptone) medium (Miller, 1992).

2.2.1 Bacterial strains

E. coli and *Y. pseudotuberculosis* strains used in this study are listed in Table 1.

Table 1. List of bacterial strains

strain	characteristics	references
***E. coli* strains**		
BL21λDE3	F$^-$ *gal met r*$^-$*m*$^-$ *lon hsdS* λ_{Lys}p*lac*UV5-T7 gene1 p*lacI*^{q} *lacI*	(Studier & Moffatt, 1986)
CC118λpir	F$^-$ Δ(*ara-leu*)7697 Δ(*lacZ*)74 Δ(*phoA*)20 *araD139 galE galK thi rpsE rpoB arfE*^{am} *recA1*, λ*pir*	(Manoil & Beckwith, 1986)
DH5αZ1	(φ80d *lacZ*ΔM15) *endA1 recA1 hsdR17 supE44 thi*$^{-1}$, *gyrA*	(Lutz & Bujard, 1997)
DH10β	F$^-$ *mcrA* Δ(*mrr-hsd*RMS-*mcr*BC) Φ80d*lacZ*ΔM15 Δ*lac*X74 *end*A1 *rec*A1 *deo*R Δ(*ara*,*leu*)7697 *ara*D139 *gal*U *gal*K *nup*G *rps*L λ$^-$	
KB2	BL21λDE3, *hns*$^-$	K. Boehme
***Y. pseudotuberculosis* strains**		
YPIII	pIB1, wild-type	(Bolin *et al.*, 1982)
YP3	pIB1, *rovA*::Tn10(60), Cm^R	(Nagel *et al*., 2001)
YP12	pVY$^-$	
YP60	pIB1, Δ*ypsI*, Kan^R	AK. Heroven
YP61	pIB1, Δ*ypsR*, Kan^R	AK. Heroven
YP62	pIB1 Δ*ypsRI*, Kan^R	AK. Heroven
YP63	pIB1, Δ*clpP*, Kan^R	AK. Heroven
YP67	pIB1, Δ*lon*, Amp^R	AK. Heroven
YP68	pIB1, Δ*clpP*, Δ*lon*, Amp^R, Kan^R	AK. Heroven
YP50	pIB1, Δ*ymoA*, Kan^R	AK. Heroven
YP53	pIB1, Δ*csrA*, Kan^R	AK. Heroven
YP72	pIB1, Δ*rovM*	K. Boehme
YP78	pIB1, Δ*lon*	AK. Heroven
YP89	pIB1, Δ*crp*	AK. Heroven

2.3 Plasmids

All plasmids used in this study are listed in Table 2. For plasmids constructed in this study the corresponding oligonucleotides are listed in Table 23.

Table 2. List of plasmids

plasmid	description	references
pACYC184	cloning vector, p15A, Cm^R, Tet^R	(Chang & Cohen, 1978)
pAKH43	pET28a, *rovM*$^+$, Kan^R	(Heroven & Dersch, 2006)
pAKH47	pGP20, *rovA-lacZ*(17)^b, Tet^R	(Heroven & Dersch, 2006)
pBAD-HisA	overexpression vector, Amp^R	Invitrogen
pGP20	protein fusion vector, ´lacZ, Tet^R	P. Gerlach
pET28a	overexpression vector, Kan^R	Novagen
pGP704	R6K cloning plasmid, Amp^R	(Miller & Falkow, 1988)
pGN17	pACYC184, *rovA-lacZ*(127)^b, Cm^R	(Heroven e*t al*., 2004)
pGN25	pACYC184, -547 bp *rovA* upstream region *rovA*$^-$ *lacZ*(17)^b, Cm^R	(Heroven *et al*., 2004))
pHT95	pQE30, *his*$_6$*-rovA*$^+$, Amp^R	H. Tran-Winkler
pHT105	pZS*24, P*LtetO-1*, Kan^R	H. Tran-Winkler
pHT123	pHT105, P*LtetO-1*::*rovA*$^+$, Kan^R	H. Tran-Winkler
pHT125	pGP704, *phoA*$^+$, Amp^R	H. Tran-Winkler
pJG23	ColEI cloning vector, *csiD*$^+$, Amp^R	J. Germer
pKD46	p*BAD gam bet exo* pSC101 on TS, Amp^R	(Datsenko & Wanner, 2000)
pUC19	overexpression vector, Amp^R	(Yanisch-Perron *et al.*, 1985)
pBAD18	overexpression vector, Amp^R	(Guzman *et al.*, 1995)
pQE30	His_6-tagging overexpression vector, Amp^R	Qiagen
pFU51	pZE12luc, Δ*luc*, *egfp*, ΔcolE1, sc101*, Amp^R	F. Uliczka
pFU69	pZE12luc, Δ*luc*, *egfp*, colE1, ΔAmp^R, Kan^R	F. Uliczka
pFU76	pZE12luc, Δ*luc*, *dsred2*, ΔcolE1, R6Kmob, Amp^R	F. Uliczka
pFU33	pZE12luc, Δ*luc*, *luxCDABE*, ΔR6Kmob, ori29807	F. Uliczka
pYPL	pPROBE-*gfp*[LVA] containing *rovA* promoter fragment from nt -622 to 170, Kan^R	(Lawrenz & Miller, 2007)
pKH1	pGP20,*lon-lacZ*(6)^b, Tet^R	this study
pKH4	pHT105, PLtetO-1::*phoA*$^+$, Kan^R	this study
pKHTS3	pBADHisA, *lon*$^+$, Amp^R	this study

pKH15	pACYC184, *rovA-lacZ*(23)[b], Cm^R	this study
pKH16	pACYC184, *rovA-lacZ*(106)[b], Cm^R	this study
pKH17	pACYC184, *rovA-lacZ*(101)[b], Cm^R	this study
pKH18	pACYC184, *rovA-lacZ*(117)[b], Cm^R	this study
pKH20	pACYC184, *rovA-lacZ*(26)[b], Cm^R	this study
pKH21	pACYC184, *rovA-lacZ*(42)[b], Cm^R	this study
pKH22	pACYC184, *rovA-lacZ*(74)[b], Cm^R	this study
pKH23	pACYC184, *rovA-lacZ*(96)[b], Cm^R	this study
pKH24	pUC19, P*rovA*, Amp^R	this study
pKH26	pHT123, P_{tet}o-1::*rovA* (E71K), Kan^R	this study
pKH31	pHT105, P_{tet}O-1::r*ovM*+, Kan^R	this study
pKH46	pFU72, $rpoS^+$, Amp^R	this study
pKH69	pFU51, P*rovA*::*rovA*, Amp^R;	this study
pKH70	pFU76, P*rovA*::*rovA-gfp*LVA, Amp^R, ΔR6Kmob, ori29807; containing *rovA* promoter fragment from -622 to +170	this study
pLW1	pET28a, $rovA^+$, Kan^R	L. Winkler
pLW2	pET28a, *rovA-his*$_6^+$, Kan^R	(Tran *et al.*, 2005)
pMB113	pQE60, *lon-his*$_6^+$, Amp^R	M. Bujara
pMB114	pHT123, ΔKan^R, Tet^R	M. Bujara
pQE60	His_6-tagging overexpression vector	Qiagen
pTAC3575	$phoA^+$, Amp^R	(Atlung *et al.*, 1991)
pZS-21	pSC101, P_{tet}*O-1* MCS-1, Kan^R	(Lutz & Bujard, 1997)
pZE12luc	colE1, P_{lac}O-1, *luc*, Amp^R	(Lutz & Bujard, 1997)

[a] the number indicates the codon of *rovA*, in which the resistance cassette has been inserted

[b] the number indicates the codon of the corresponding gene fused to *lacZ*

[c] the number indicates the codon of the corresponding gene fused to *phoA*

2.4 Oligonucleotides

All oligonucletide primers used in this study were purchased from Metabion and are listed in Table 3.

Table 3. List of oligonucleotides.

description	sequence	restriction site[a]
Pr. 153	GCCGCGTCTAGATGCCGCCTTCCTGCAACTCG	*XbaI*
Pr. 158	GCCGCGGTCGACTGCCGCCTTCCTGCAACTCG	*Sal*I
Pr. 492	GCGGCGCTGCAGCGCGCCAAACGCGAACTAATCG	*Pst*I
Pr. I175	GCGGGGGAGCTCAACCCTGAGCGTTCCGAAC	*Sac*I
Pr. I274	GCATACGAATTCGGTGAATAATTAACCAGATTGTCC	*Eco*RI
Pr. I275	GCATACAAGCTTCGGAACGCTCAGGGTTCATAGAG	H*ind*III
Pr. I369	GGGCGGCTGCAGCTATTTTGGCGCTACTGGCTG	*Pst*I
Pr. I553	GAGGAGCAATTGCACCATCACCATCACCATGAATCGACATTAGG	none
Pr. I554	CCTAATGTCGATTCATGGTGATGGTGATGGTGCAATTGCTCCTC	none
Pr. I572	TCTGACTGCCGGAACCGTG	none
Pr. I675	GCGATCGTCGACTCCCCCCAAGTATTTCTTTGCG	*Sal*I
Pr. I676	GTGGCTGTCGACGTTCGATTATCGGTGAAGACTG	*Sal*I
Pr. I751	CGCATCGTCGACACGGTTTCAGCCGATGGTCAATTAATG	*Sal*I
Pr. I752	CGCATCGTCGACAACGATTAATGTTATACAAAGTAACCC	*Sal*I
Pr. I753	CGCATCGTCGACAACCTTTTTCCTCCAGTGATCCAAG	*Sal*I
Pr. I754	CGCATCGTCGACAAGACTGTTCCGTTAATTTAATTCA C	*Sal*I
Pr. I802	GGTTAGAACCTTGGATCAACTGAAGGAAAAAGGTTTAATCACACGG	none
Pr. I803	CCGTGTGATTAAACCTTTTTCCTTCAGTTGATCCAAGGTTCTAAC	none
Pr. I978	GCGCGCGGTACCAAAGTGAAGAATACACTATGACAAATGC	*Kpn*I
Pr. I979	GCGCGCATCGATAAACAGATTACTTAATCTTCATCACC	*Cla*I
Pr. II207	GCGCGCGTCGACTAATTGCATGAAGATGCTGATTTTGATG	*Sal*I
Pr. II208	GCGCGCGCGGCCGCTTACTTCGGCCGTTAGCAGTGGC	*Not*I

[a] restriction sites are underlined

2.5 Methods

2.5.1 General microbiological methods

2.5.1.1 Sterilisation

All autoclavable solutions and plastic materials were sterilized for 20 min at 121°C and 2 bar overpressure. Non-autoclavable solutions were sterile filtered (pore diameter 0.2 µm). Glass materials like pipettes, flasks and tubes were sterilized at 180°C in a dry heat sterilizer.

2.5.1.2 Measurement of cell density

The cell density of bacterial cultures was determined photometrically by measurement of the optical density at 600 nm wavelength in a spectrophotometer. An OD_{600} of 1 corresponds to approximately 1 x 10^9 cells/ml (Sambrook *et al.*, 1989) and a protein content of 150 µg (Miller, 1992).

2.5.1.3 Fluorescence Microscopy

To visualize the *rovA* expression in single cells during exponential growth phase, the *Y. pseudotuberculosis* wild-type strain harboring a *rovA-gfp* promoter fusion plasmid was grown overnight at different temperatures and diluted 1:50 with fresh, prewarmed LB medium. Subsequently, cells were grown for additional 3 to 4 hrs at the corresponding temperature. Samples for fluorescence microscopy were taken every hour and cells were fixed with 4% PFA (pH 7.4). After washing three times with PBS cells were examined using a Zeiss Axio Observer.D1m Fluorescence Microscope (Zeiss). Images were taken by using AxioVison software (Zeiss) and adjusted by Adobe Photoshop (Adobe).

2.5.1.4 FACS analysis

For analysis of heterogeneous *rovA* expression, a *rovA-gfp* expressing YPIII strain (YPIII pKH70) was grown overnight at different temperatures and diluted 1:50 with fresh, pre-warmed LB medium. Subsequently cells were grown for additional 3 hrs at the corresponding temperature. Samples for flow cytometric analysis were taken and 1 ml cells were fixed with 1 ml 4% PFA (pH 7.4, in PBS) for 15 min. After washing 3 times with PBS *rovA-gfp* expression of at least 100.000 cells were analyzed using a BD LSRII (BD Biosciences) flow cytometer. Data were recorded with FACS Diva software (BD Biosciences) and further analyzed with FlowJo 9.0.2.

2.5.2 General molecular biology techniques

For general work with DNA, such as PCR, vector restriction analysis, ligations and transformations common procedures were applied (Sambrook *et al.*, 1989). Kits for plasmid preparation, PCR purification and gel extraction were used from Qiagen, restriction enzymes and ligases were purchased from New England Biolabs or Promega. PCRs were performed in 50 µl aliquots using Taq or Long Amp Taq polymerase (New England Biolabs) or Phusion polymerase (Finnzymes) according to the manufacturer's instructions.

2.5.2.1 Construction of plasmids

2.5.2.1.1 Construction of *rovA-lacZ* fusion plasmids

For construction of the *rovA-lacZ* fusion plasmids pKH15-23 used for identification of the RovA portion which is sensitive to proteolysis, different PCR fragments of the 5'-region were amplified with the upstream primer 158 and the downstream primers 86, 572, I676, I675 and I751-I754 and inserted in frame into the vector pGN17, harboring the *lacZ* sequence. Successful cloning was proven by DNA sequencing.

2.5.2.1.2 Site directed mutagenesis

In order to introduce point mutations into plasmid pHT123, site directed mutagenesis was performed using the QuikChange® Site-Directed Mutagenesis Kit (Stratagene) according to the manufacturer's instructions. After *Dpn*I digestion plasmids were

transformed into chemically competent *E. coli* K-12 cells and streaked out on plates with the appropriate antibiotics. Successful mutagenesis was proven by DNA sequencing.

2.5.2.2 Electro Mobility Shift Assays (EMSA)

For DNA-binding studies, purified RovA or RovM proteins were dialysed against the DNA-binding buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 5 mM dithiothreitol, 5% glycerol, 10 mM NaCl, 1 mM $MgCl_2$, 100 mg/ml BSA). Equimolar amounts of PCR fragment, carrying the *rovA* or *inv* regulatory region was mixed with the DNA binding buffer and increasing amounts of RovA or RovM. The reaction mixture was incubated for 20 min at 25°C or 37°C and subsequently separated in a 4% polyacrylamide gel and stained with ethidium bromide. The following fragments were amplified with indicated primer pairs: Fragment inv-1 of the *inv*-promoter, primer pair I266/I267; fragment inv-2, primer pair I264/I265 and fragment inv-3, primer pair 304/I263. Fragment rovA-1 of the *rovA* promoter region containing two RovA binding sites, primer pair 153/237; fragment rovA-2 pimer pair 153/296 and the *rovA*-promoter fragment rovA-3 harboring the RovM, primer pair 302/201. As a negative control, a 350 bp fragment from the *E. coli csiD* gene was amplified with primers 132/133 and included in the assay.

For determination of the apparent dissociation constant K_d a constant concentration of PCR fragments inv-1, inv-2, rovA-1 or rovA-3 were incubated with increasing concentration of RovA or RovM protein and subjected to EMSA. Bands were quantified and the concentration of protein was plotted against the percentage of bound DNA. The K_d was defined as the protein concentration required for half-maximal binding.

Table 4. List of primers used for amplification of EMSA promoter fragments

primer	sequence
Pr. 132	GGCTACGAAATGAGCATCGC
Pr. 133	ACGATTTGTACTCCAGCGCC
Pr. 153	GCCGCGTCTAGATGCCGCCTTCCTGCAACTCG
Pr. 201	GAGTCCAGTCTATTTTGGG
Pr. 237	GGGTATTGCACCGTTATCTAC
Pr. 296	GCCGCGGGATCCATTAACCTAATACGAGTATCC
Pr. 302	GTGCTAACGACAATGAC

Pr. 304	GAAATAAAAACCGCAGCAGAG
Pr. I263	GCATTTGAAATCACCTTGACC
Pr. I264	GATATTCTCACCTGCTTAG
Pr. I265	GAAAACCATCATAATTTAAATG
Pr. I266	CACAGAGGTAGACGTATC
Pr. I267	GATTTATTGTAACTAAGCAGGTG

2.5.2.3 Northern Blot

To identify the amounts of *rovA*-mRNA when expressed under control of the P_{tet} promoter Northern blot analysis was performed. Overnight cultures of *E. coli* strain DH5αZ1 pHT123 were grown overnight or to exponential growth phase (OD_{600} of 0.4-0.6) in the presence or absence of 0.1 µg/ml AHT at 25°C and 37°C. Approximately 2.5 ml of culture was taken, mixed with 0.2 volume of stop solution (5% water-saturated phenol, 95% ethanol) and immediately frozen in liquid nitrogen. After thawing on ice, bacteria were harvested by centrifugation (2 min, 14.000 rpm, 4°C), and RNA was isolated using the SV total RNA purification kit (Promega) as described by the manufacturer. RNA concentration and quality were determined by measurement of A_{260} and A_{280}.

Total cellular RNA was separated on 0.7% MOPS (20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA, pH 7) agarose gels, transferred onto a Nylon membrane (Roche) in 20 x SSC (3 M NaCl, 0.3 M sodium citrate, pH 7) and UV cross-linked. Hybridization with DIG-labeled riboprobes and membrane was conducted using the DIG luminescent Detection kit (Roche) according to the manufacturer's instructions. The *rovA* transcripts were detected with a DIG-labeled PCR fragment (DIG-PCR nucleotide mix, Roche) with primer pair 122/150.

Table 5. Primers used for detection of *rovA* transcripts

primer	sequence
Pr. 122	GCGGCGGGTACCTTGGAATCGACATTAGGATCTG
Pr. 150	GCGCCTGCAGTTATTACTTAGTTTGTAATTG

2.5.3 Biochemical methods

2.5.3.1 Overexpression and purification of the *Y. pseudotuberculosis* RovA, RovM and Lon proteins

His-tagged proteins were overexpressed in *E. coli* BL21λDE3, native RovA in the *E. coli* strain KB2. Overnight cultures of the strains harboring the overexpression plasmids pLW1 (*rovA*$^+$), pLW2 (*rovA-his*$_6^+$), pHT95 (*his*$_6$*-rovA*$^+$), pAKH43 (his$_6$-*rovM*$^+$), pKHTS3 (*his*$_6$*-lon*$^+$) or pMB113 (*lon-his*$_6^+$) were diluted 1:100 in LB and grown for 2 hrs at 37°C. Subsequently, protein production was induced by adding 100 µM IPTG or 0.2% arabinose and grown for 4 hrs or to an OD_{600} of 3-4.

His-tagged proteins were purified using a Ni-NTA sepharose (Qiagen) column. Cells containing the overexpressed proteins were harvested by centrifugation, resuspended in lysis buffer I (10 mM Tris-HCl pH 8.0, 300 mM NaCl, 10 mM imidazole) and disrupted using a French®Press (SLM Aminco). After removal of cell debris and insoluble compounds the extracts were incubated with Ni-NTA sepharose (1 h at 4°C shaking) and subjected to protein purification (BioRad). The column was washed 10 times with washing buffer I (50 mM Tris-HCl pH 8.0, 300 mM NaCl, 60 mM imidazole) and the protein was eluted in 5 fractions with elution buffer I (50 mM Tris-HCl pH 8.0, 300 mM NaCl, 250 mM imidazole).

Native RovA was purified with dsDNA cellulose (Amersham). Cells were resuspended in lysis buffer II (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 5 mM DTT, 5% glycerol) and disrupted using a French®Press (SLM Aminco). After removal of cell debris and insoluble compounds by centrifugation the extract was incubated with dsDNA cellulose for 1 h at 4°C. After sedimentation of the cellulose and removal of the supernatant the material was washed once with washing buffer IIa (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 5 mM DTT, 5% glycerol, 50 mM NaCl) and twice with washing buffer IIb (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 5 mM DTT, 5% glycerol, 100 mM NaCl). The protein was eluted in two fractions with elution buffer II (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 5 mM DTT, 5% glycerol, 300 mM NaCl).

Purity of the proteins was determined by SDS-PAGE and Coomassie staining and was estimated to be >95%.

Protein concentration was determined by using the Protein Assay Kit II (BioRad). The procedure was followed according to the manufacturer's instruction.

2.5.3.2 SDS polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

SDS-PAGE was performed according to Laemmli (Laemmli, 1970) in vertical electrophoresis chambers (BioRad, Mini-Protean II). Depending on the protein size 12-18% polyacrylamide separating gels (pH 8.8) with 4% stacking gels (pH 6.8) were used. Samples were prepared by adding SDS-sample buffer (60 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 3% β-mercaptoethanol, 0.005% bromophenol blue, H_2O) and a subsequent heat denaturation at 95°C for 5 min. Gels were either stained with Coomassie™ Brilliant Blue G250 or used for Western blot analysis.

For Western blot analysis proteins were transferred to an Immobilon PVDF membrane (Millipore) by electroblotting for 50 min at 100 V. The membrane was blocked for 1 h in TBSTM (5% dried milk powder, 20 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.05% Tween 20) or overnight in TBSTB (3% BSA, 20 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.05% Tween 20). RovA and RovM were detected using polyclonal antibodies derived from rabbit. Invasin was detected by using a monoclonal mouse derived antibody. Incubation with the primary antibody was performed for 1 to 2 hrs, followed by washing with TBST (20 mM Tris-HCl pH 7.5, 100 mM NaCl, 0.05% Tween 20) and incubation with an anti-rabbit or anti-mouse alkaline phosphatase conjugated antibody or with an anti-rabbit horseradish peroxidase conjugated antibody. Blots incubated with alkaline phosphatase conjugated antibodies were developed using NBT and BCIP, blots incubated with horseradish peroxidase were developed by using the Western Lightning ECL II Kit (Perkin Elmer).

2.5.3.3 *In vivo* protein stability assay

For *in vivo* protein stability assays cultures were grown to exponential or stationary growth phase at 25°C. 20 ml culture was centrifuged at 4000 rpm for 7 min and the remaining pellet was resuspended in 20 ml of fresh medium pre-incubated at 25°C or 37°C. Protein synthesis was stopped by adding 200 µg/ml chloramphenicol or 50 µg/ml tetracycline. Subsequently cultures were further incubated at 25°C or 37°C for additional 90 min. Samples of 1 ml were taken at the indicated time points. Whole cell extracts of equal amounts of bacteria were prepared by adding SDS-sample buffer followed by heat denaturation at 95°C for 5 min and incubation with Benzonase (Merck) for 30 min at 37°C. Protein degradation was visualized by Western blotting.

To identify a putative RovA stabilizing factor cultures were grown over night or to exponential growth phase at 25°C in LB, M9, MMA or DMEM/F12 (3:1, Biochrom) medium. Cells were centrifuged, the supernatant was sterile filtered and supplied with the appropriate additives. Alternatively stationary phase medium was pretreated at different conditions. Subsequently, exponentially grown cells were resuspended in the supernatant and protein stability assays were performed as described above.

2.5.3.4 *In vitro* protein stability assay

Purified Lon protease and RovA were dialysed against the reaction buffer (50 mM Tris-HCl pH 8.0, 4 mM DTT, 10 mM $MgCl_2$). 3 µM of Lon protease was mixed with 12.5 µM RovA or α-casein. For ATP regeneration 80 µg/ml creatine kinase and 20 mM creatine phosphate were added. The reaction was started by supplying 4 mM ATP. Reaction mixtures were separated and incubated at 25°C or 37°C. The reaction was stopped at certain time points by adding SDS-sample buffer and heat denaturation at 95°C for 5 min. Samples were loaded on a polyacrylamide gel and degradation was analyzed by Coomassie staining.

To test whether an additional factor is required for *in vitro* proteolysis *E. coli* strain BL21λDE3 pLW1 (*lon*$^-$ *rovA*$^+$) was grown to an OD_{600} of 0.6 at 25°C and 37°C, resuspended in reaction buffer and disrupted using a French®Press (SLM Aminco). Purified Lon protease (3 µM) was added to the cell extract with or without ATP and the ATP regenerating system. Degradation of RovA was detected by SDS-PAGE and Coomassie staining.

2.5.3.5 CD spectroscopy

For CD spectroscopy 0.16 mg/ml RovA, 0.4 mg/ml purified RovM and 0.16 mg/ml lysozyme in CD buffer (10 mM NaH_2PO_4 pH 8.0, 10 mM NaCl, 5 mM DTT, 1 mM $MgCl_2$) were used. Spectra were recorded on a Jascow J-810 spectrometer in a 1 mm silica glass cuvette using a thermostated cell holder. Each spectrum was the result of five successive spectra normalized against the CD buffer. Spectra were recorded starting at 25°C. After 10 min equilibrium delay the temperature was raised to 37°C. After re-shifting the temperature again to 25°C, 2 hrs of equilibration time were allowed. Secondary structure estimation was performed using the Jascow spectrometer J-810 analysis software, which makes use of the Yang algorithm.

Temperature stability was determined by using a temperature slope from 20°C to 60°C for RovA, from 20°C to 70°C for RovM and from 20°C to 85°C for lysozyme with a temperature slope of 2°C/min at 222 nm wavelengths. 0.5 mg/ml RovA, 0.2 mg/ml RovM and 0.5 mg/ml lysozyme were used.

2.5.3.6 β-galactosidase activity assay

Specific β-galactosidase activity was determined according to Miller (Miller, 1992). 200 µl of diluted stationary grown culture or exponentially grown culture were lysed with one drop of 0.1% SDS and two drops of chloroform, followed by 5 min incubation at RT. 1.8 ml Z-buffer (100 mM sodium phosphate pH 7.0, 10 mM KCl, 1 mM $MgSO_4$) was added. The reaction was started with 0.4 ml ONPG (4 mg/ml in H_2O) and stopped at appropriate yellow coloration with 1 ml Na_2CO_3. After centrifugation (Eppendorf, MiniSpin, 14.000 rpm, 10 min) the absorption of a 200 µl sample was determined at 405 nm in an ELISA microtiter plate reader (BioRad). The specific enzyme activity was determined in duplicates and calculated as follows:

$$\text{specific activity } [\mu\text{mol}\cdot(\text{mg}\cdot\text{min})^{-1}] = OD_{405}\cdot 6.75^{*} \ (OD_{600}\cdot\Delta t\cdot V)^{-1}$$

OD_{405}:	optical density of stopped reaction
6.75:	extinction coefficient of cleaved ONPG
OD_{600}:	optical density of bacterial culture
V:	culture volume
Δt:	time from start to stop of enzyme reaction

2.5.3.7 Alkaline phosphatase activity assay

Specific alkaline phosphatase activity was determined according to (Manoil & Beckwith, 1986). 1 ml of stationary or exponentially grown culture was washed twice with 250 mM Tris-HCl. For cell lysis two drops of 0.1% SDS and two drops of chloroform were added to 0.5 ml of washed cells and incubated for 10 min at RT. 1.5 ml of 250 mM Tri-HCl was added. The reaction was started with 0.4 ml PNPP (4 mg/ml in H_2O) and stopped at appropriate yellow coloration with 0.2 ml 1 M K_2HPO_4. After centrifugation (Eppendorf, MiniSpin, 14.000 rpm, 10 min) the absorption of the sam-

ple was determined at 405 nm using a microtiter plate reader (BioRad). The specific enzyme activity was determined in duplicates and calculated as follows:

$$\text{specific activity } [\mu\text{mol}\cdot(\text{mg}\cdot\text{min})^{-1}] = OD_{405}\cdot 6.46\cdot(OD_{600}\cdot\Delta t\cdot V)^{-1}$$

OD_{405}:	optical density of stopped reaction
6.46:	extinction coefficient of cleaved PNPP
OD_{600}:	optical density of bacterial culture
V:	culture volume
Δt:	time from start to stop of enzyme reaction

2.5.3.8 ThermoFluor analysis

ThermoFlour analysis is a fluorescence-based thermal stability assay monitoring the effect of ligand binding to temperature-dependent protein unfolding. Thereby, binding of a hydrophobic fluorophore to hydrophobic patches of an unfolding protein creates a fluorescence signal. The resulting curve is used to determine the melting temperature of the protein.

To determine the binding affinity of RovA to putative ligand molecules the effect of varying ligand concentrations on protein T_m was measured by ThermoFluor analysis in a Real Time PCR machine (Eppendorf). 2 mg/ml RovA was mixed with 100 x Sypro Orange (Invitrogen) and different concentrations of ligand in reaction buffer (50 mM Tris-HCl pH 8, 100 mM NaCl) and filled into 96-well plates. Plates were sealed and measured at an excitation of 495 nm and an emission of 610 nm. For each plate the temperature was increased from 10°C to 90°C in 0.5°C increments.

The protein T_m was plotted against the ligand concentration and the dissociation constant K_d was calculated by fitting the data with the following equation:

$$T = T_0 + \frac{\frac{L_0 + P_0 + K_d}{2} \pm \sqrt{\frac{(L_0 + P_0 + K_d)^2}{4} - L_0 P_0}}{P_0}(T_{max} - T_0)$$

T melting temperature

T_0 melting temperature without ligand

T_{max} melting temperature in saturated state

L_0 initial ligand concentration

K_d dissociation constant

P_0 initial protein concentration

3 Results

3.1 Temperature and growth phase dependent regulation of RovA

3.1.1 *rovA* expression is controlled on a post-transcriptional level

The *rovA* gene in *Y. pseudotuberculosis* is regulated via a complex regulatory network involving autoregulation, regulation by H-NS, RovM, YmoA and the Csr-system. It could be shown that RovA is maximally expressed at 25°C in stationary growth phase, whereas minimal expression was observed at 37°C in exponential growth phase (Heroven *et al*., 2004, Nagel *et al*., 2001). However, none of the known regulators in the network contributes to this temperature and growth phase dependent regulation. Previous experiments showed a temperature-dependent regulation of an *inv-phoA* fusion in *E. coli* when *rovA* was expressed under the control of the *lac* promoter (Nagel *et al*., 2001), suggesting a post-transcriptional regulation mechanism for *rovA* expression.

To further address this hypothesis *rovA* was expressed from a tetracycline inducible promoter (P_{tet}) in *E. coli* strain DH5αZ1 pHT123 (Figure 3-1A, B). DH5αZ1 encodes the tetracycline repressor gene *tetR* and therefore allows a tight transcriptional control by the tetracycline analogue anhydrotetracycline (AHT). Cells were grown at 25°C and 37°C at both exponential and stationary growth phase and RovA synthesis was induced with 0.1 μg/ml AHT. RovA-dependent *inv-phoA* and *inv-lacZ* fusions were still expressed under *rovA* inducing conditions (25°C, stationary phase) and repressed under *rovA* non-inducing conditions (37°C, exponential phase) as observed in alkaline phosphatase and β-galactosidase activity assays. To exclude temperature and growth phase dependency of the promoter, *phoA* was expressed under control of P_{tet}, showing no induction under these conditions (Figure 3-1A).

Additionally, Northern blot analysis was performed under analogue conditions. *rovA* mRNA expressed from the P_{tet} promoter was found in identical amounts at 25°C and 37°C in exponential and stationary growth phase (Figure 3-1C). These results strongly suggest that temperature and growth phase dependent *rovA* expression is controlled on a post-transcriptional level.

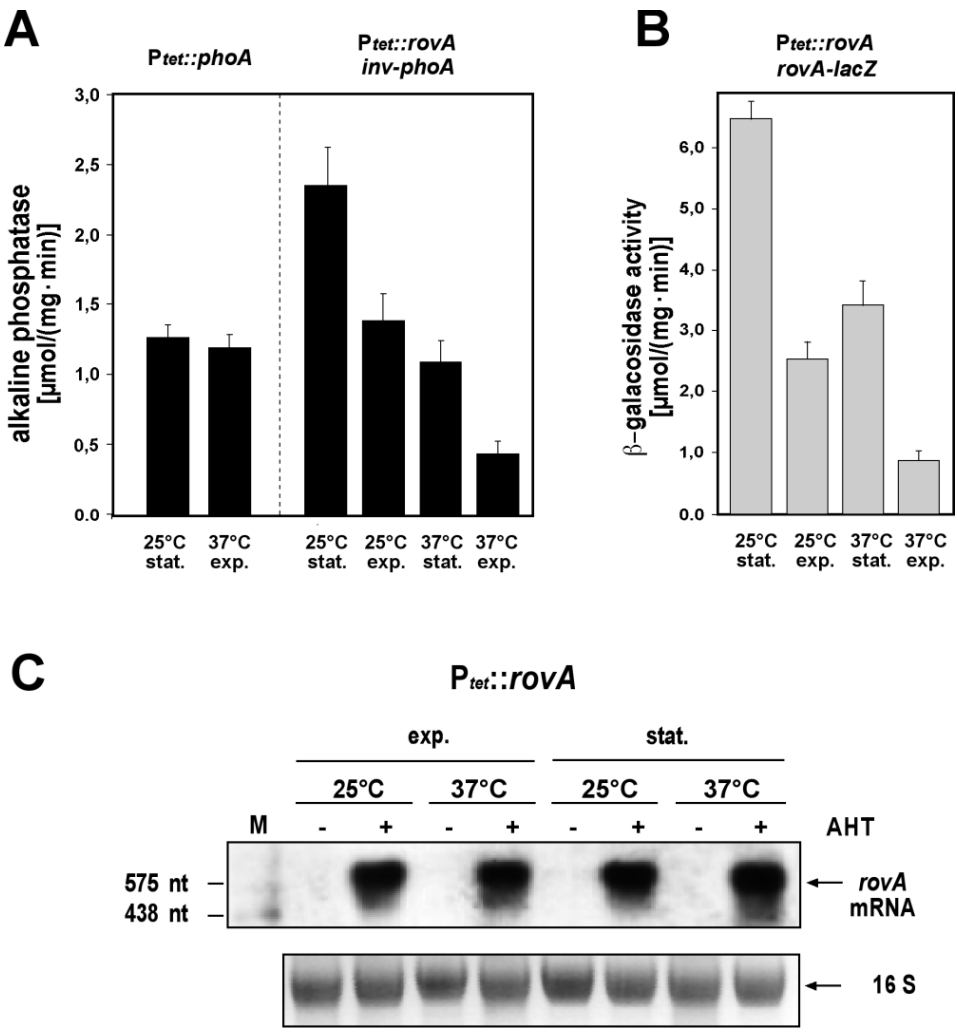


Figure 3-1. Temperature and growth phase-dependent expression of *rovA*. (A, left panel) Expression of *phoA* under control of the P_{tet} promoter. **(A, right panel)** Expression of an *inv-phoA* fusion in *E. coli* strain DH5αZ1 pHT123. **(B)** Expression of a *rovA-lacZ* fusion in *E. coli* strain DH5αZ1 pHT123. *rovA* is expressed under control of the P_{tet} promoter. Strain DH5αZ1 encodes the *tet* repressor gene *tetR*. Strains were grown overnight or to exponential growth phase at 25°C or 37°C, *rovA* expression was induced with anhydrotetracycline (AHT, 0.1 µg ml^{-1}). Alkaline phosphatase and β-galactosidase activity was determined in triplicates in three independent experiments. **(C)** Northern blot analysis. *E. coli* strain DH5αZ1 pHT123 was grown with (+) or without (-) AHT (0.1 µg ml^{-1}). Total RNA was prepared, separated on a 0.7% agarose gel, transferred onto a Nylon membrane and probed with a digoxigenin-labeled PCR fragment encoding the *rovA* gene. 16 sRNA served as control. RNA marker is loaded on the left, arrows indicate the *rovA* mRNA.

3.1.2 RovA binds to DNA in a temperature-dependent manner

Genetic screenings to identify cellular components responsible for temperature dependent *rovA* expression and attempts to find temperature induced protein modifica-

tions (e.g. phosphorylation) were not successful (Hien Tran-Winkler, unpublished). Therefore, it was hypothesized that RovA itself might act as a temperature sensor whose conformation and function might be influenced by temperature. Such temperature induced conformational changes should lead to a modified DNA-binding capacity of the protein to the *inv* and *rovA* regulatory regions. To test this, electro mobility shift assays (EMSA) were performed at 25°C and 37°C with different *inv* or *rovA* promoter fragments including either one or two *rovA* binding sites with increasing amounts of protein. To exclude unspecific binding of RovA to DNA a fragment of the *E. coli csiD* gene was used as a control.

As shown in Figure 3-2A, the DNA-binding ability of RovA is strongly reduced at 25°C compared to 37°C. Much higher amounts of RovA were necessary to cause a shift of DNA at 37°C at both promoter fragments inv-1 and inv-2. For the inv-3 fragment containing both *rovA* binding sites of the *inv*-promoter, a very moderate shift could be observed at 37°C, whereas two RovA-DNA complexes occurred at 25°C (Figure 3-2A). To quantify this effect the apparent dissociation constant K_d was calculated. This constant is commonly used to describe the ability of a protein to bind to a ligand or to DNA. For the *inv*-promoter binding site I and II a K_d of about 32±5 nM and 45± nM, respectively, was calculated at 25°C (Figure 3-2B). For the same binding sites K_d values of 183±24 nM and 109±23 nM were measured at 37°C.

A similar thermo-induced reduction of DNA-binding was seen with *rovA* promoter fragments containing only one or two *rovA* binding sites (Figure 3-3A). K_d values of 46±3 nM and 25°C and 178±5 nM at 37°C were determined for binding site I, showing a 4-fold reduction of binding affinity at 37°C (Figure 3-3B). Interestingly, reduced DNA-binding was observed when both RovA binding sites were present in the *inv* promoter as well as in the *rovA* promoter fragments. The amount of higher molecular weight RovA-DNA complexes was strongly reduced in the *inv* or *rovA* promoter fragments harboring two RovA binding sites. This suggests that specifically cooperative binding of RovA to DNA is strongly impaired at 37°C.

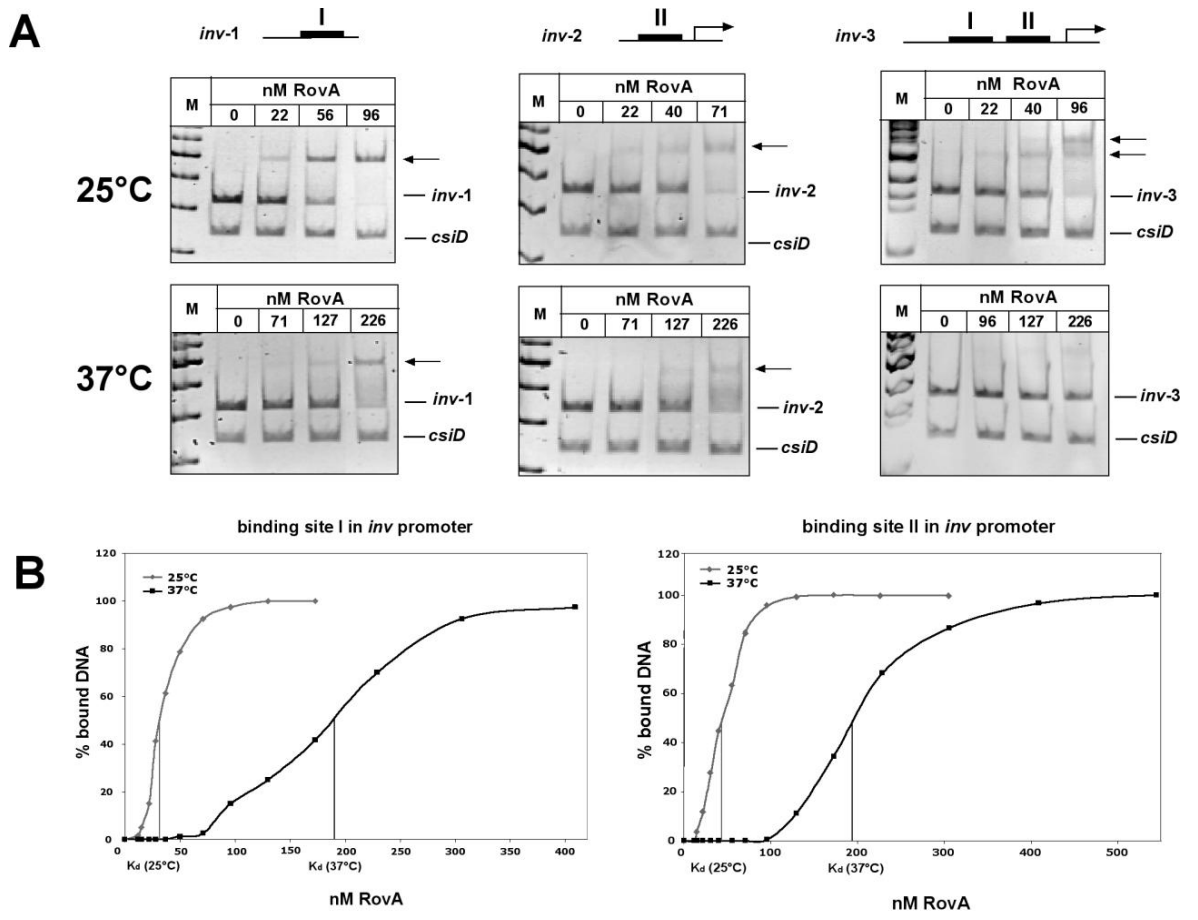


Figure 3-2. Interaction of RovA with the *inv* regulatory region at 25°C or 37°C. (**A**) Promoter fragments of the *inv* regulatory region harboring one or both RovA binding sites (black boxes I and/or II) were incubated with rising amounts of purified RovA protein at 25°C or 37°C. The DNA-RovA complexes were separated on a 4% polyacrylamide gel. A non-specific probe containing an unrelated sequence (*csiD* promoter of *E. coli*) was included as negative control. (**B**) A constant concentration of DNA fragments, harbouring site I or II of the *inv* promoter region, were incubated with different increasing concentrations of RovA and subjected to band shift assays. The DNA bands were quantified and the K_d was defined as the protein concentration required for half-maximal binding. K_d values were determined as the average of at least four independent experiments.

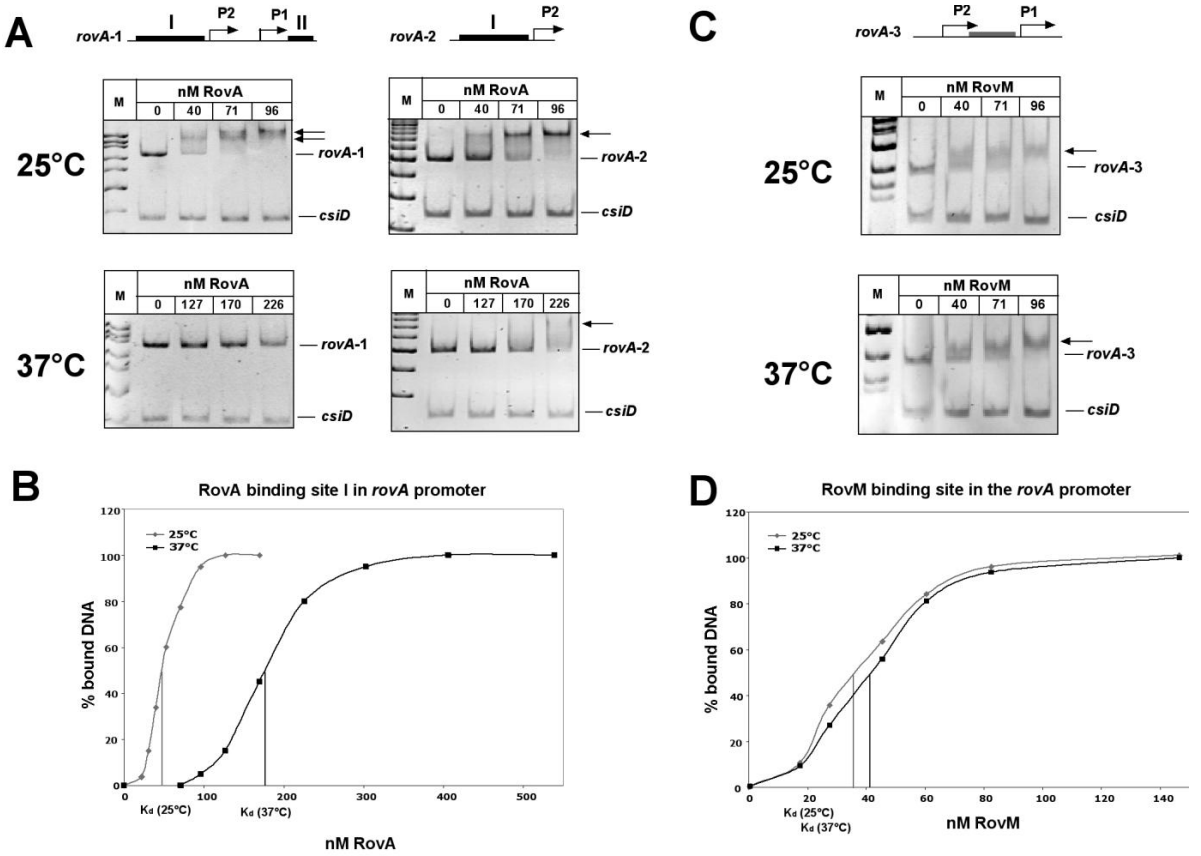


Figure 3-3. Interaction of RovA or RovM with the *rovA* regulatory region at 25°C or 37°C. Promoter fragments of the *rovA* regulatory region harbouring RovA binding sites (black boxes) or the RovM binding site (grey box) were incubated with rising amounts of purified RovA protein (**A**) or RovM (**C**). The DNA-protein complexes were separated on a 4% polyacrylamide gel. A non-specific probe containing an unrelated sequence (*csiD* promoter of *E. coli*) was included as negative control. A constant concentration of DNA fragments, harbouring RovA binding site I or the RovM binding site of the *rovA* promoter region, were incubated with different increasing concentrations of RovA (**B**), or RovM (**D**) and subjected to band shift assays. The DNA bands were quantified and the K_d was defined as the protein concentration required for half-maximal binding. K_d values were determined as the average of at least four independent experiments.

To further analyze if reduced DNA-binding at higher temperatures is only restricted to RovA, DNA-binding of RovM to the *rovA* promoter region was examined (Figure 3-3C, D). RovM binds to a region between bp -80 and -47 within the *rovA* promoter region and was shown to form higher molecular complexes with the DNA (Heroven & Dersch, 2006). In contrast to RovA no significant changes in DNA-binding affinity were detected for RovM at all tested concentrations. A K_d of 36± 6 nM was determined at 25°C and a slightly higher K_d of 41±3 nM at 37°C (Figure 3-3D). These re-

sults clearly demonstrate that temperature-induced reduction of DNA-binding activity is explicitly limited to RovA.

3.1.3 Temperature induces conformational changes of RovA

In order to elucidate the mechanism of temperature dependent DNA-binding, possible conformational changes within the protein were further investigated. Structural analysis of RovA revealed a highly α-helical structure in which the first α-helix of one RovA monomer inserts between the α_5 and α_6 helix of the other monomer, thus creating a well-packed dimer interface. Interacting coiled-coil domains are known to be sensitive to temperature changes. Consequently, the loss of DNA-binding capacity at 37°C could be mediated by temperature induced conformational changes within the protein. To address this, Circular Dicroism spectroscopy was performed. Spectra of highly purified RovA (0.16 mg/ml) were recorded between 200 nm and 250 nm at 25°C. After heating the sample up to 37°C, the protein was re-cooled to 25°C (Figure 3-4A). The RovA spectra clearly show a change of conformation when temperature is increased to 37°C. Cooling down the protein to 25°C leads to the initial conformation, indicating that the overall structure is only partially altered. A change of conformation seems to lead to loss of the α-helical structures and to an increase of β-sheets (Table 6).

Table 6. Secondary structure of RovA at different temperatures. Secondary structure components of purified RovA were estimated using the Yang algorithm.

Secondary structure	25°C	37°C	25°C after recooling
α-helices	53.5%	41.5%	52.3%
β-sheets	15.5%	25.6%	16.2%
turn	0%	0%	0%
random	31.0%	32.9%	31.5%

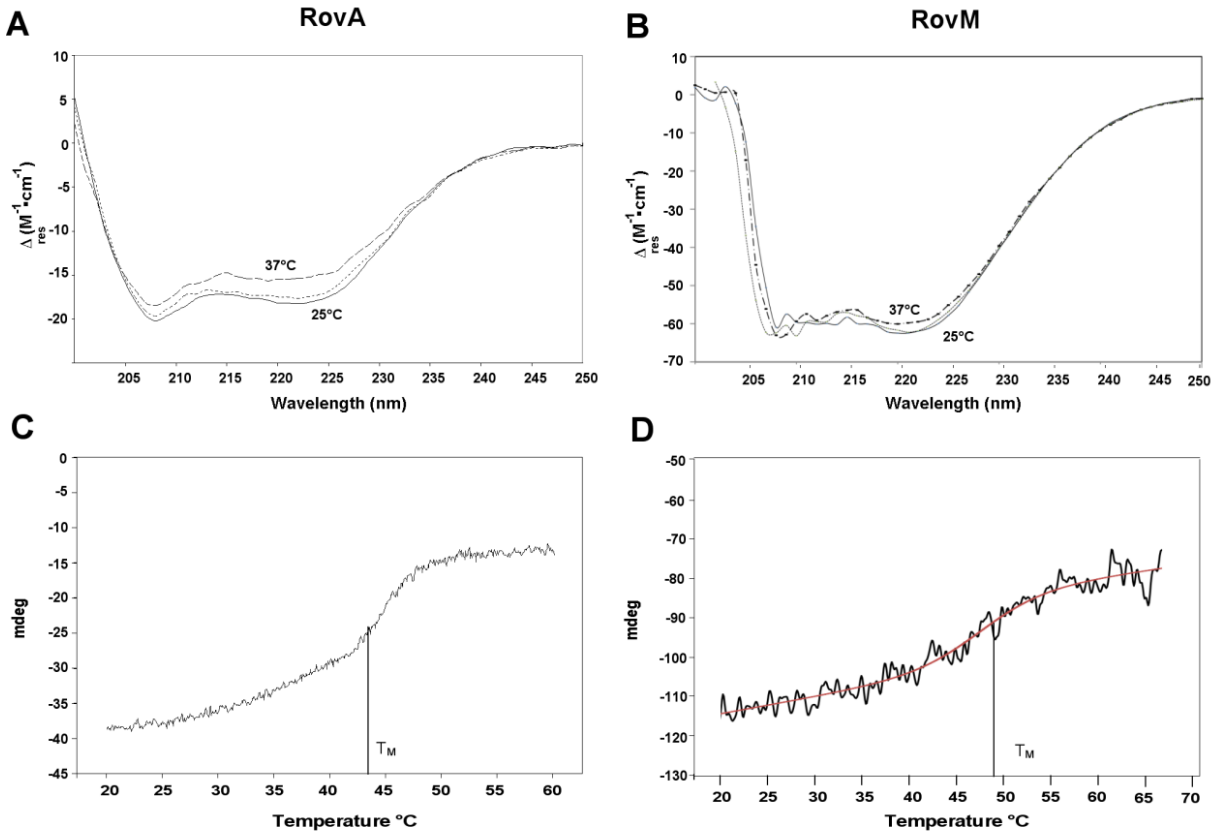


Figure 3-4. Analysis of RovA and RovM conformation. CS spectra, De ($M^{-1}cm^{-1}$) versus wavelength of RovA (0.16 mg/ml) (**A**) and RovM (0.4 mg/ml) (**B**) as function of temperature (25°C, solid line; 37°C broken line; 37°C and cooling to 25°C dotted line). Thermal stability of the RovA (**C**) and RovM (**D**) proteins. The temperature of the purified RovA protein solution was increased from 20°C to 60°C with a temperature slope of 2°C/min. For the RovM protein temperature was increased from 20°C to 70°C. The denaturation curve was recorded at fixed wavelength of λ=222nm. The melting points (T_m) were calculated using the Jascow spectra analysis software.

In contrast, control spectra of RovM (Figure 3-4B) and lysozyme (data not shown) did not show any detectable conformational changes.

To gain more information about protein stability, temperature scans between 20°C and 60°C at a fixed wavelength at 222 nm and a temperature slope of 2°C/min were performed. The melting point (T_m) of RovA was determined to be 44.5°C. Furthermore, a pronounced gradient between 20°C and 40°C was observed, indicating conformational changes within this temperature range (Figure 3-4C). Stability curves with the control proteins RovM (Figure 3-4D) and lysozyme (data not shown) proceeded without a comparable slope to the melting points of 48°C and 74°C, respectively.

Taken together, these results indicate, that temperature-dependent DNA-binding abilities are a consequence of reversible conformational changes between 25°C and 37°C.

3.1.4 Stability of RovA is dependent on temperature and growth phase

Expression of the *rovA* gene from a temperature independent promoter should lead to similar levels of protein at 25°C and 37°C. However, transcription from the AHT inducible P_{tet} promoter at different temperatures and growth phases in a *Y. pseudotuberculosis rovA* mutant (YP3 pHT123) (Figure 3-5A) and in *E. coli* (DH5αZ1 pHT123) (Figure 3-5B) led to lower RovA levels at 37°C compared to 25°C. In contrast, no difference in the protein content at different temperatures was observed when expressing *rovM* from the P_{tet} promoter in *E. coli* (DH5αZ1 pKH31) (Figure 3-5C). Therefore, it was assumed that the temperature induced conformational changes may influence protein stability. To test this, the influence of growth temperature on the stability of RovA was investigated in *Y. pseudotuberculosis* YPIII and *E. coli* DH5αZ1 pHT123 after blockage of protein synthesis (Figure 3-6A-F).

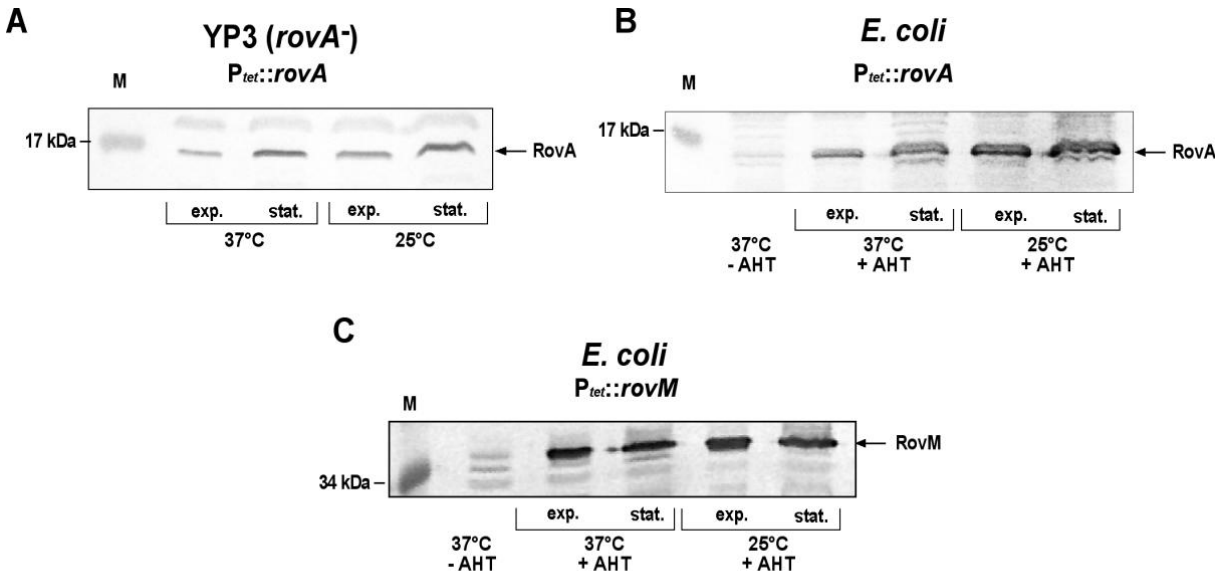


Figure 3-5. Analysis of intracellular *rovA* and *rovM* expression under the control of the tetracycline promoter (P_{tet}). *Y. pseudotuberculosis* strain YP3 pHT123 (**A**), *E. coli* strain DH5αZ1 pHT123 (**B**) and *E. coli* strain DH5αZ1 pKH31 (**C**) were grown overnight or to exponential phase at 25°C or 37°C in the presence of the inducer AHT (0.1 µg ml^{-1}). Whole cell extracts from the cultures were prepared and analyzed by Western blotting using a polyclonal antibody directed against RovA or RovM.

Y. pseudotuberculosis YPIII and *E. coli* DH5αZ1 pHT123 were grown to exponential or stationary phase at 25°C to ensure expression of native RovA. Subsequently, protein synthesis was blocked and cultures were divided and incubated either at 25°C or 37°C. As presented in Figure 3-6A/C, stability of RovA could be observed at 25°C during stationary phase for at least 90 min whereas at 37°C slightly reduced levels of RovA were found. In the exponential growth phase RovA was rapidly degraded at 37°C and no protein could be detected 30 min after blockage of protein synthesis. In contrast, only slightly reduced RovA levels were observed at 25°C (Figure 3-6B/D). Yet, no differences in RovM levels were detected under the same conditions (Figure 3-7A-F).

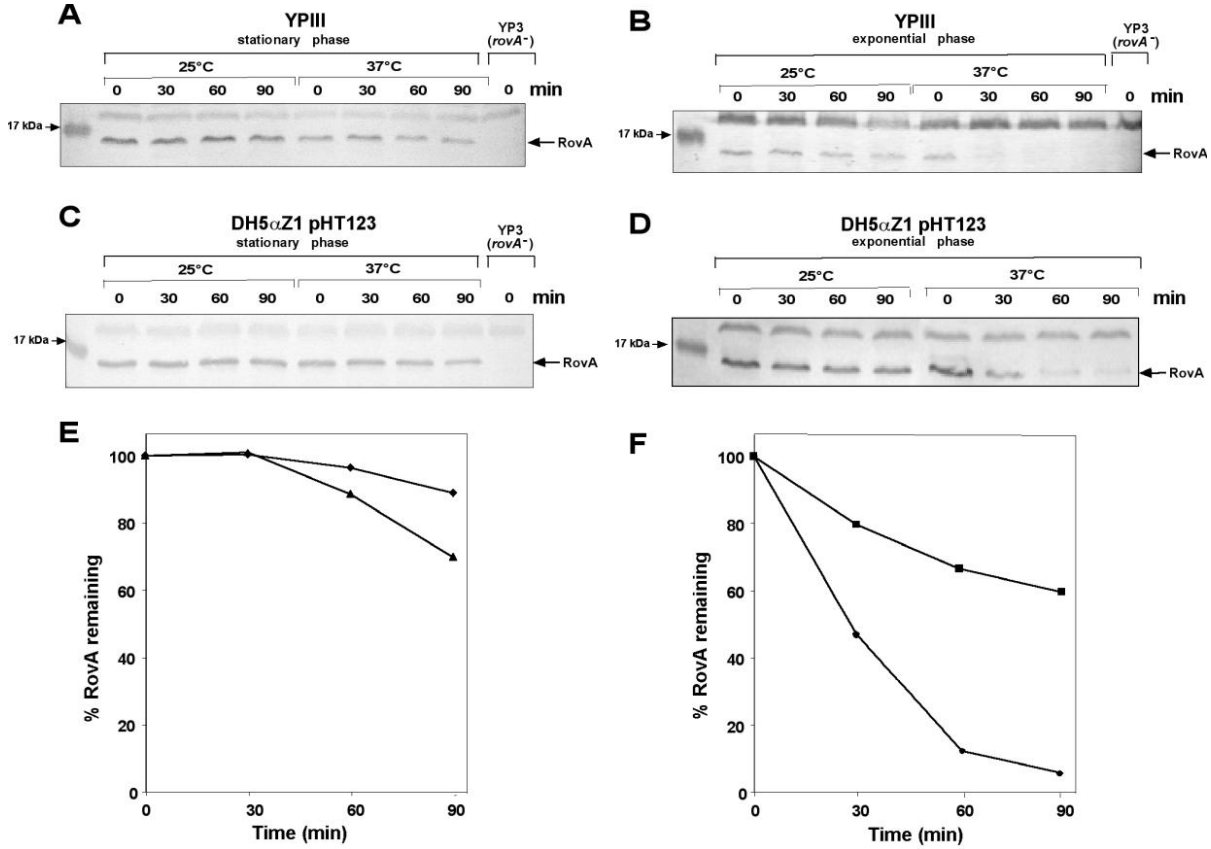


Figure 3-6. Stability of RovA during exponential and stationary phase at 25°C and 37°C. Cultures of *Y. pseudotuberculosis* strain YPIII (wt) (**A-B**) and *E. coli* strain DH5αZ1 pHT123 (**C-F**) were grown overnight or to exponential phase (OD_{600} = 0.3-0.4) at 25°C before chloramphenicol (200 µg/ml) was added. The cultures were divided and incubated at 25°C or 37°C for additional 90 min. Aliquots of the cultures were analyzed by Western blotting. A whole cell extract from the *rovA* mutant strain YP3 grown overnight at 25°C was used as a control. (**E, F**) Protein bands of DH5aZ1 pHT123 were quantified using the BioRad analysis software 'Quantity One' 4.6.2 and set into relation to sampling at time point zero (◆ 25°C, stationary phase; ▲ 37°C, stationary phase; ■ 25°C, exponential phase; ● 37°C, exponential phase).

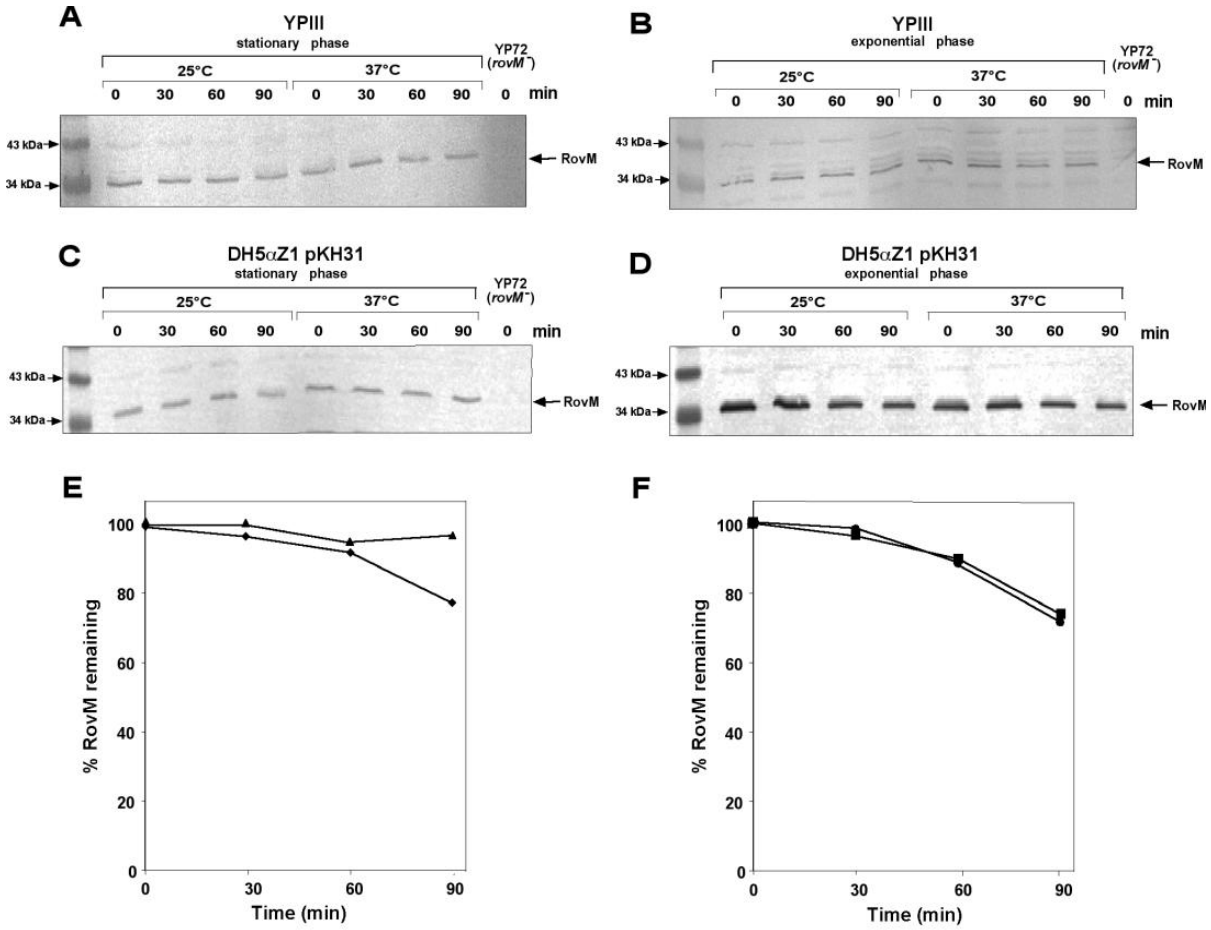


Figure 3-7. Stability of RovM during exponential and stationary growth phase at 25°C and 37°C. Cultures of *Y. pseudotuberculosis* strain YPIII (**A/B**) and *E. coli* strain DH5αZ1 pKH31 (**C-F**) were grown overnight or to exponential growth phase at 25°C. After addition of chloramphenicol to stop protein synthesis cultures were divided and either incubated at 25°C or 37°C for 90 min. Samples were taken at indicated time points and analyzed by Western blotting. As a control whole cell extracts from the *rovA* mutant strain YP72 were used. Protein bands were quantified using the BioRad analysis software Quantity One 4.6.2 and set into relation to time point zero. (◆ 25°C, stationary phase; ▲ 37°C, stationary phase; ■ 25°C, exponential phase; ● 37°C, exponential phase).

Taken together, these data suggest that RovA is not only inactivated at higher temperatures but also rapidly degraded under non inducing conditions. Furthermore, the post-transcriptional regulation of RovA appears to be conserved in *E. coli* and *Y. pseudotuberculosis*.

3.1.5 RovA stability is affected by the ATP-dependent proteases Lon and ClpP

Degradation of regulatory proteins is often mediated by the Lon and ClpP proteases, the most important and best studied proteases in bacteria. Thus, it was very likely that one or both types of proteases are involved in RovA proteolysis. To prove this, *Y. pseudotuberculosis lon*, *clpP* and *lon/clpP* deletion mutants were constructed. Subsequently, expression of *rovA-lacZ* fusions as well as RovA levels in the mutant strains were compared to the wild-type at 25°C and 37°C. To this purpose, a plasmid containing a *rovA-lacZ* fusion (pAKH47) was transformed into all strains and *rovA* was expressed either from its own promoter (P_{rovA}::*rovA*, Figure 3-8A) or from the P_{tet} promoter (P_{tet}::*rovA*, Figure 3-8B) in stationary growth phase.

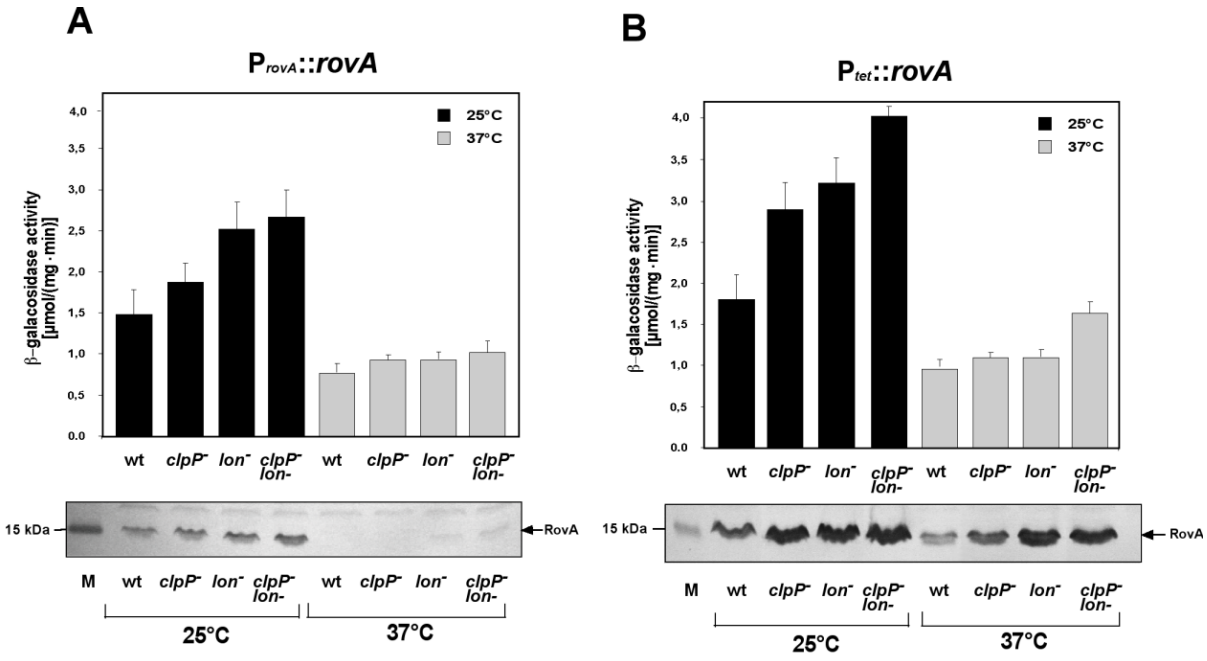


Figure 3-8. *rovA* expression in *Y. pseudotuberculosis* wild-type and protease mutants at 25°C and 37°C. Expression of a *rovA-lacZ* fusion on pAKH47 was analyzed in *Y. pseudotuberculosis* YPIII (wt) and in the *clpP, lon* and *clpP/lon* mutant strains (YP63, YP67 and YP68), respectively. *rovA* was either expressed from its own promoter (P_{rovA}::*rovA*) (**A**), or in the presence of the *rovA* expression plasmid pHT123 (P_{tet}::*rovA*) (**B**). Cultures were grown at 25°C and 37°C in LB medium overnight and β-galactosidase activity was determined. The data represent the average ± SD from at least three different experiments each done in duplicate (upper panel). Furthermore, whole cell extracts of equal amounts of the bacteria were prepared and visualized by Western blotting

These data clearly show higher RovA levels in the *lon* mutant and the *clpP/lon* mutant at 25°C compared to the wild-type when *rovA* was expressed from its own pro-

moter. In contrast, only a slight increase of RovA levels in the *clpP* mutant was observed, suggesting that RovA is mainly degraded by the Lon protease. Very low amounts of RovA could be observed at 37°C when *rovA* was expressed from its own promoter (Figure 3-8A), even in the absence of proteases. Similar results could be observed in β-galactosidase activity assays employing a plasmid-based *rovA-lacZ* fusion. The absence of RovA at 37°C and the low expression of the *rovA-lacZ* fusion in all strains can be explained by the fact that DNA-binding of the protein to its own promoter region is strongly reduced at this temperature and therefore prevents autoregulation.

To avoid autoregulation the experiment was repeated under the same conditions with *rovA* expressed under control of the P_{tet} promoter (Figure 3-8B). Synthesis of similar levels of RovA could be observed at both temperatures with slightly higher amounts of RovA in the mutants. Moreover, higher expression of the RovA-dependent *rovA-lacZ* fusion at 25°C compared to *rovA* expression under its own promoter was demonstrated. At 37°C very little activation of *rovA* transcription was found, although RovA levels were similar to 25°C. These results imply that the thermo-induced conformational changes and the related reduced DNA-binding capacity play a more important role in *rovA* autoregulation compared to proteolysis.

3.1.6 RovA proteolysis is mainly mediated by the Lon protease

To have a closer look on Lon- and ClpP-dependent RovA degradation, RovA was expressed from plasmid pHT123 in *Y. pseudotuberculosis* YPIII or in the protease mutants YP63, YP67 and YP68. Plasmid pHT123 harbors *rovA* under the control of the P_{tet} promoter to prevent autoregulation and ensure efficient RovA production in the exponential growth phase. Cells were grown overnight or to exponential growth phase at 25°C. After stop of protein synthesis the cultures were either kept at 25°C or shifted to 37°C. Samples were taken at indicated time points and visualized by Western blotting.

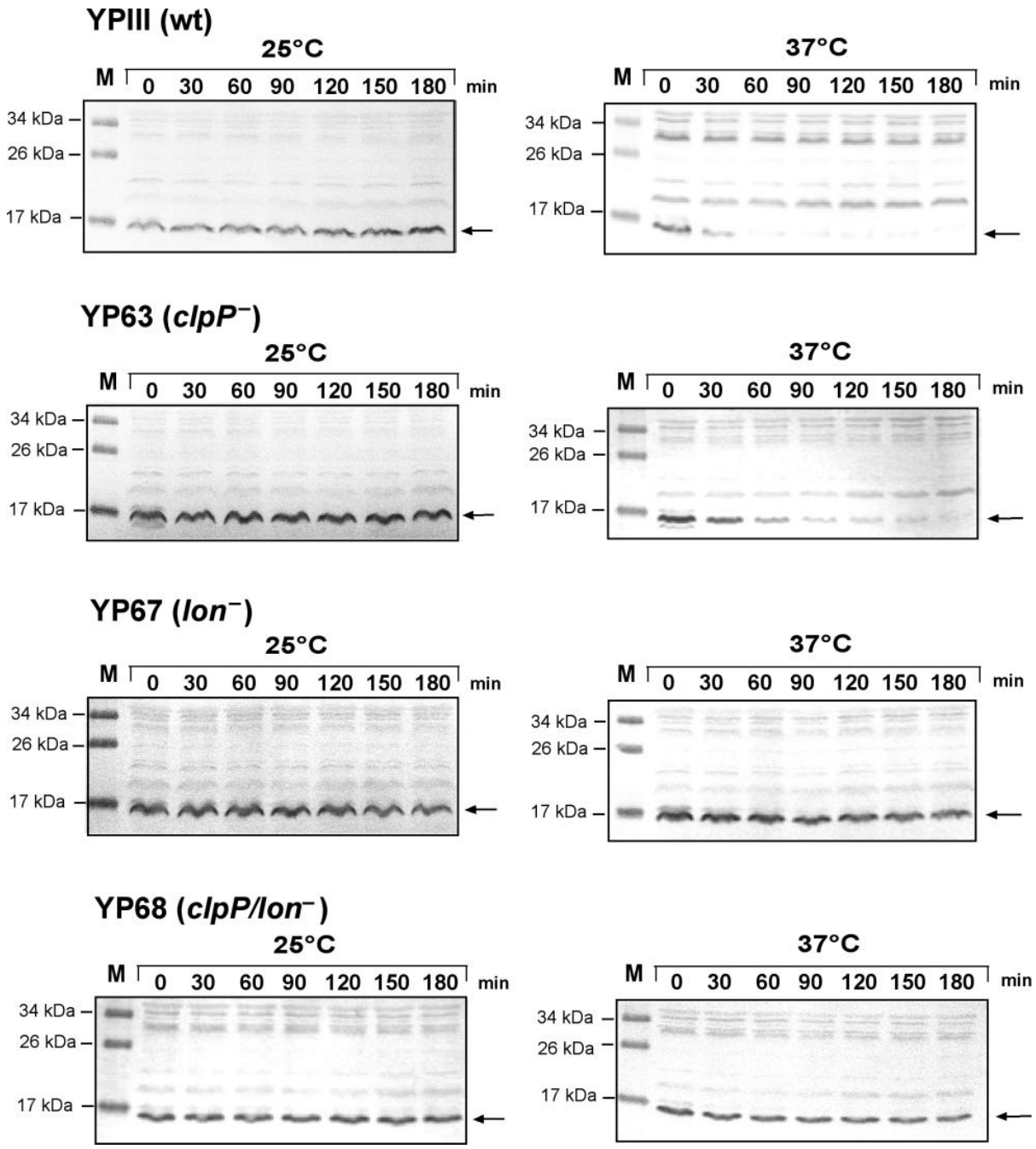


Figure 3-9. Stability of RovA during exponential growth phase at 25°C and 37°C in *Y. pseudotuberculosis* YPIII, YP63 (Δ*clpP*), YP67 (Δ*lon*), YP68(Δ*lon/clpP*). Strains harboring the P_{tet}::*rovA* expression plasmid pHT123 were grown to exponential growth phase (OD_{600} = 0.3-0.5) at 25°C. To stop protein synthesis chloramphenicol (200 µg/ml) was added. Cultures were divided and either incubated at 25°C or at 37°C for 180 min. Samples were taken at the indicated time points and analyzed by Western blotting. On the left side a prestained protein marker is added. The arrows on the right indicate the RovA protein.

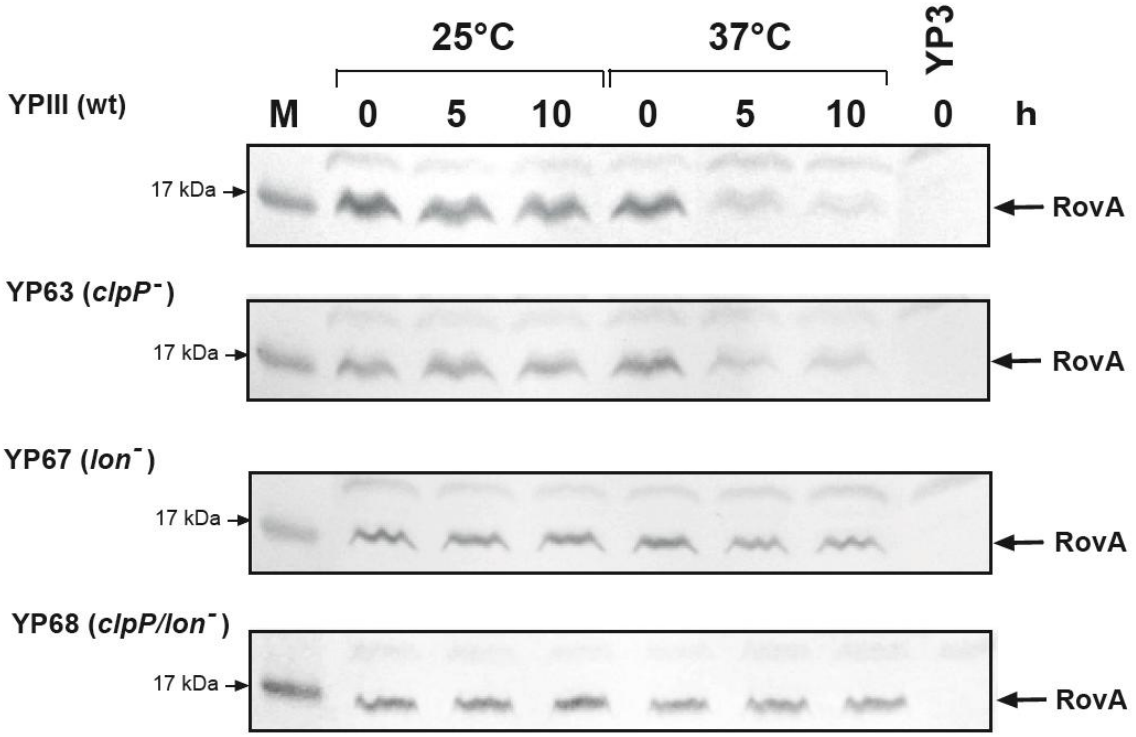


Figure 3-10. Stability of RovA during stationary growth phase at 25°C and 37°C in *Y. pseudotuberculosis* YPIII, YP63 (Δ*clpP*), YP67 (Δ*lon*), YP68(Δ*lon/clpP*). Strains harboring the P_{tet}::*rovA* expression plasmid pHT123 were grown overnight at 25°C. To stop protein synthesis chloramphenicol (200 µg/ml) was added. Cultures were divided and either incubated at 25°C or at 37°C for additional 10 hrs. Samples were taken at the indicated time points and analyzed by Western blotting. As a control a *rovA* mutant (YP3) was included. On the left side a prestained protein marker is added.

In exponential growth phase (Figure 3-9 and S1, Supplements) RovA is rapidly degraded in the wild-type at 37°C ($t_{1/2}$ = 30 min) and very low amounts of the protein are observed 1 h after blocking protein synthesis. Similar results could be shown for the *clpP* mutant at 37°C even though slightly higher RovA levels could be detected compared to the wild-type. In contrast, hardly any degradation of RovA could be found in the Lon mutant and in the double mutant at 37°C. Significantly less RovA degradation could be observed in the wild-type and in the mutant strains grown at 25°C. The same degradation pattern was shown in stationary phase cultures even though proteolysis was strongly delayed (Figure 3-10). RovA was barely detectible 5 hrs after stop of protein synthesis in the wild-type and the *clpP* mutant, whereas the protein remained completely stable in the *lon* and lon/*clpP* mutant. In summary, these results clearly show that RovA degradation is predominantly mediated by the Lon protease while ClpP only contributes in a very small extent to RovA proteolysis.

3.1.7 Identification of the RovA domain sensitive to proteolysis

In order to identify the amino acid motive required for protease recognition, the stability of different RovA-LacZ hybrid proteins was determined. Being resistant to Lon degradation, β-galactosidase fused to different RovA fragments and subsequent analysis of hybrid stability should reveal the location of amino acids necessary for proteolysis.

The expression plasmids pGN17, pKH16, pKH21-23 encoding RovA-LacZ fusion proteins with different portions of the RovA protein were transformed into *Y. pseudotuberculosis* strain YPIII and transformants were grown to exponential growth phase at 25°C. Subsequently, protein synthesis was stopped and cultures were incubated at 37°C for 90 min. Samples were taken at indicated time points and visualized by Western blotting. Finally, the amount of degraded protein was quantified (Figure S1, Supplements).

Degradation assays of a RovA-LacZ fusion protein containing the first 127 amino acids of RovA ($RovA_{1-127}$-LacZ) demonstrated a protein half-life comparable to the wild-type RovA. Protein turnover of the same fusion protein was completely blocked in a *Y. pseudotuberculosis lon* mutant (Figure 3-11A). Also RovA-LacZ hybrids constituted of the 106 or 96 N-terminal amino acids were still subjected to proteolysis (Figure 3-11B, C) whereas hybrids containing the N-terminal 74 amino acids remained stable and were still visible 90 min after blocking protein synthesis (Figure 3-11D). In contrast, chimera containing the first 42 amino acids of RovA remained completely stable under the same conditions (Figure 3-11E).

Taken together, these results indicate that the amino acids between residue 42 and 96 are important for regulated RovA proteolysis. These residues are located in the proximity of the RovA-DNA-binding domain. Previous results demonstrated that DNA-binding at 37°C is significantly reduced. Under the same conditions RovA proteolysis is enhanced. Hence, binding of the protein to DNA quite likely prevents its degradation by the Lon protease. To test this possibility, a plasmid harboring both RovA-binding sites in the *rovA* promoter region (pKH24) and an empty vector (pUC19) were transformed into YPIII. Stability of the native RovA protein was determined at 37°C in exponential growth phase. Compared to the vector control, presence of multiple RovA binding sequences led to increased RovA levels in the cell (Figure 3-12A). Nevertheless, RovA degradation was not completely abolished. Additionally, a plas-

mid harboring RovA with an amino acid substitution in the winged-helix domain (RovA E71K) that was known to diminish DNA-binding was introduced into YPIII *(Tran et al*., 2005), revealing reduced half-life of the protein (Figure 3-12B). Taken together, these results indicate that binding to DNA prevents proteolysis by making RovA less accessible fort the protease.

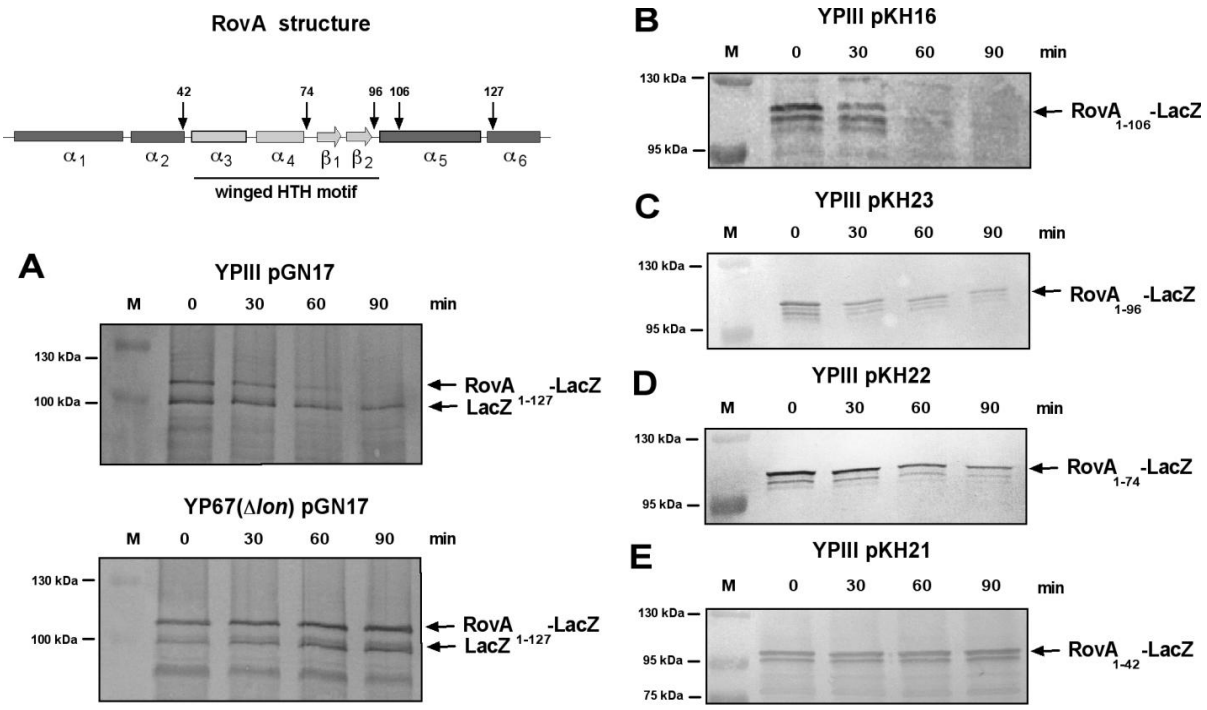


Figure 3-11. Stability analysis of RovA-LacZ hybrids. Analysis was performed as described. Aliquots of the cultures were removed at the indicated time points and analyzed by Western blotting with a polyclonal antibody directed against β-galactosidase (**A**) or RovA (**B-E**). A prestained molecular weight marker is loaded on the left. The structure of the RovA protein is given in the upper panel on the left. The predicted α-helical domains implicated in dimerization are given in dark grey, the two α-helices of the HTH motif are indicated by light grey boxes, and the two β-sheets of the winged HTH DNA-binding motif are given in light grey arrows. The numbers illustrated with arrows indicate the last amino acid fused to β-galactosidase.

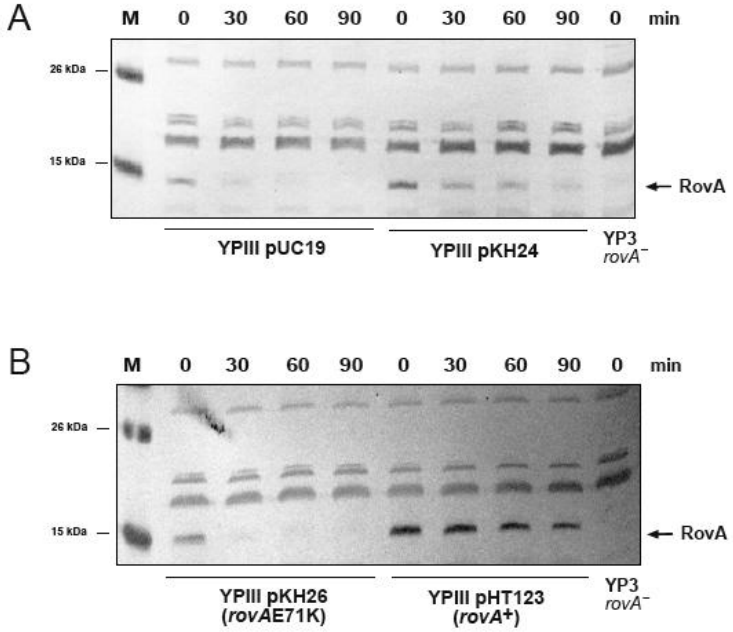


Figure 3-12. Prevention of proteolysis by DNA-binding. (A) Stability of RovA at 37°C in exponential phase in *Y. pseudotuberculosis* YPIII pUC19 and YPIII pKH24, harboring the binding sites of the *rovA* promoter. **(B)** Stability of YPIII pKH26 and YPIII pHT123 expressing the mutant RovA E71K and RovA wild-type, respectively. Analysis was performed as described. Aliquots of the cultures were removed at the indicated time points and analyzed by Western blotting. A prestained molecular weight marker is loaded on the left.

3.1.8 *In vitro* degradation by the Lon protease

Analysis of RovA stability in *Y. pseudotuberculosis* revealed Lon as the main protease mediating RovA degradation *in vivo*. To show a direct interaction of the regulatory protein and the protease an *in vitro* stability assay was established, consisting of purified RovA, purified Lon, ATP and an ATP-regenerating system and the reaction buffer. For regeneration of ATP, creatine kinase (80 µg/ml) and creatine-phosphate (20 mM) were added. The reaction mix was incubated at either 25°C or 37°C. To prove the activity of purified Lon protease, α-casein was introduced into the assay. As shown in Figure 3-13A, B α-casein is rapidly degraded at 25°C and 37°C, demonstrating a temperature-independent activity of the Lon protease. Quantification of native RovA levels after *in vitro* degradation revealed, that protein degradation occurs very slowly at 37°C whereas no degradation could be observed at 25°C (Figure 3-13C, D). Moreover, assays employing N- or C-terminally His-tagged variants of Lon from *E. coli* and *Y. pseudotuberculosis* displayed similar results (data not shown).

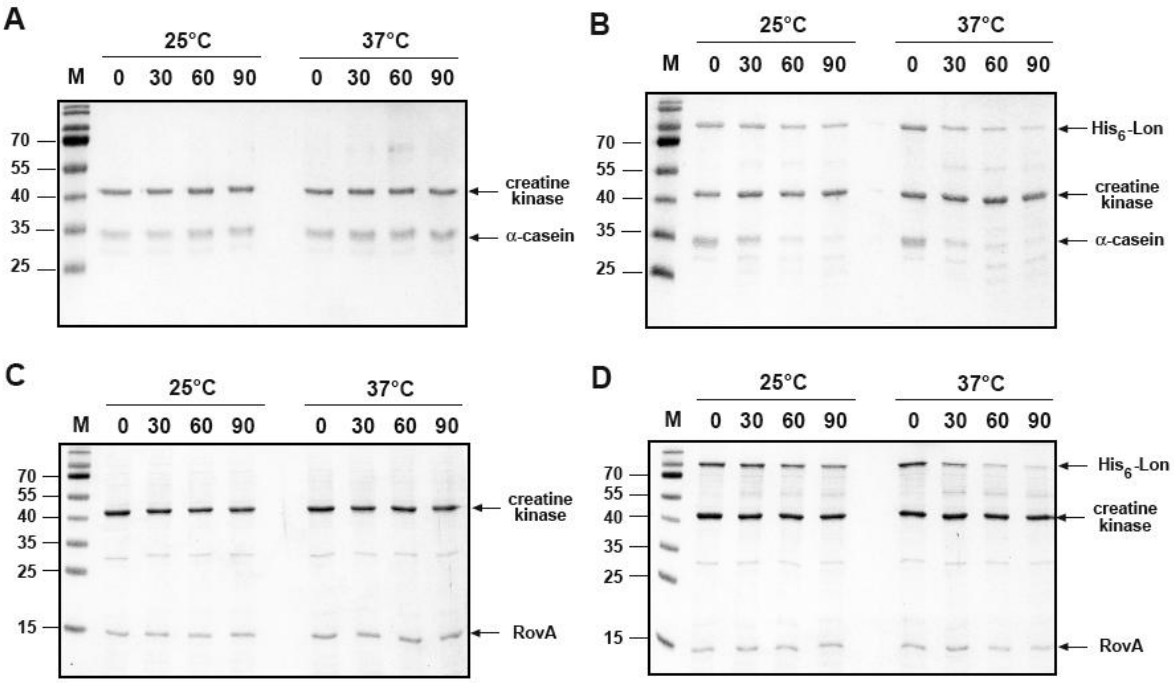


Figure 3-13. *In vitro* degradation of α-casein and RovA by Lon. α-casein and purified RovA were incubated at 25°C and 37°C in the presence of ATP and an ATP regeneration system consisting of creatine phosphate and creatine kinase without **(A, C)** or with **(B, D)** purified Lon. The degradation reactions were performed as previously described (2.5.3.4). An aliquot was removed at the indicated time points; proteins were separated by SDS-PAGE and visualized by Coomassie staining.

Slow degradation of RovA observed *in vitro* suggests the involvement of accessory factors or chaperones that may affect RovA recognition or enzymatic activity of Lon. To test this hypothesis, Lon-mediated degradation of RovA was measured in a crude extract of *E. coli* BL21 (*lon*$^-$) cells after addition of the purified Lon protease in the presence and absence of ATP and the ATP regenerating system (Figure 3-14A, B). RovA degradation was observed in the presence of Lon and ATP but not in the absence of one of them (Figure 3-14C). In addition, RovA remained completely stable in extracts from cells grown under conditions that normally do not result in protein degradation (Figure 3-14D). These findings strongly suggest the necessity of one or more accessory factors to ensure efficient degradation of RovA *in vitro*.

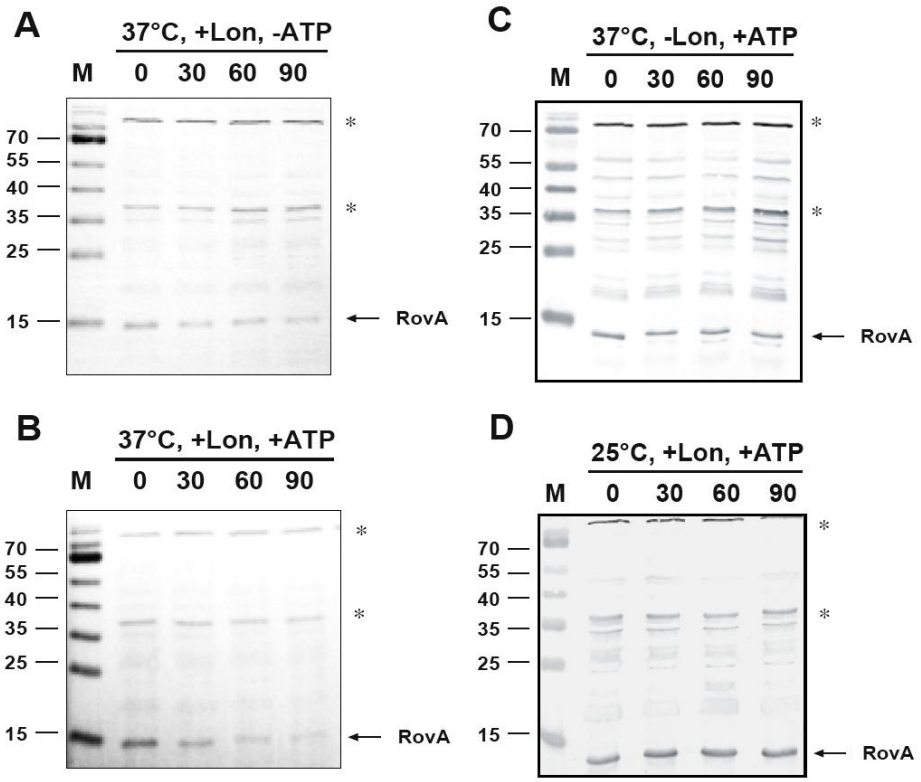


Figure 3-14. *In vitro* degradation of RovA by Lon. Purified Lon and RovA protein were added to whole cell extracts of *E. coli* BL21 (*lon*$^-$) in the absence **(A)** and presence of ATP and the ATP regeneration system **(B)** at 37°C. As a control, cell extracts were incubated in the presence of ATP without Lon **(C)** and at 25°C in the presence of ATP and Lon **(D)**. The degradation reactions were performed as described previously (2.5.3.4). An aliquot was removed at the indicated time points and RovA was visualized by Western blotting with an anti-RovA antibody. * indicates protein bands that interacted non-specifically with the RovA antibody and which were used for loading controls.

3.1.9 Temperature-dependent synthesis of the Lon protease

The previous results showed that the Lon protease is quickly degrading RovA *in vivo* at 37°C but not at 25°C. As the protease is part of the heat shock regulon and its synthesis increases when the bacteria are exposed to higher temperatures (42°C) (Phillips *et al.*, 1984, Goldberg, 1992) the question was raised if Lon synthesis is increased at temperatures where RovA is preferentially degraded i.e. at 37°C. Therefore, *lon* expression in *Y. pseudotuberculosis* YPIII was analyzed at 25°C and 37°C in exponential and stationary growth phase using a *lon-lacZ* fusion and Western blotting to analyze Lon levels (Figure 3-15). Analysis of the *lon-lacZ* fusion revealed a much higher *lon* expression at 37°C compared to 25°C. Furthermore, slightly ele-

vated protein levels were detected in stationary growth phase. In agreement with these results, low amounts of Lon were found in cell extracts of *Y. pseudotuberculosis* at 25°C in both growth phases whereas significantly higher amounts of the protease could be detected at 37°C. Consequently, it is likely that temperature dependent *lon* expression also contributes to accelerated RovA degradation at higher temperatures. However, the mechanism of growth phase dependent degradation of RovA at 37°C remains unclear and other mechanisms seem to promote RovA degradation under these conditions.

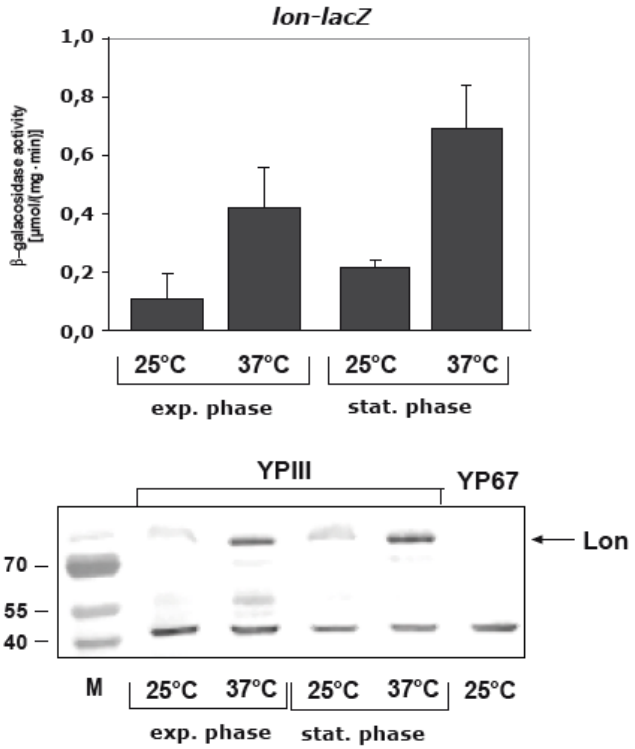


Figure 3-15. Temperature and growth phase dependent regulation of *lon* expression. Expression of *lon* was analyzed at 25°C and 37°C at exponential and stationary growth phase in a *Y. pseudotuberculosis* YPIII pKH1 strain, harboring a *lon-lacZ* fusion. β-galactosidase activity was measured and is represented as the mean of three different experiments, each done in duplicate (upper panel). For Western Blot analysis whole cell extracts of equal amounts of bacteria were prepared and visualized using a Lon polyclonal antibody from *E. coli*. Whole cell extracts of a *Y. pseudotuberculosis* mutant (YP67) were used as a control. A prestained molecular weight marker is loaded on the left (lower panel).

3.1.10 RovA stabilization occurs in late stationary growth phase

Previous experiments demonstrated that RovA is rapidly degraded by the Lon protease at 37°C in exponential growth phase, whereas proteolysis is strongly reduced in stationary growth phase. In this study, results of *in vivo* and *in vitro* experiments suggested an additional component that is involved in growth phase dependent RovA proteolysis. The nature and physiological properties of this factor are unknown. To further characterize the factor, the stage in the growth phase at which the factor is affecting RovA degradation was investigated. Therefore, RovA stability assays were performed with *Y. pseudotuberculosis* strain YPIII grown to different optical densities at 25°C. Protein biosynthesis was stopped by addition of 200 µg/ml chloramphenicol before incubating the cells at 37°C. Samples were taken at the indicated time points and RovA amounts were visualized by Western blotting.

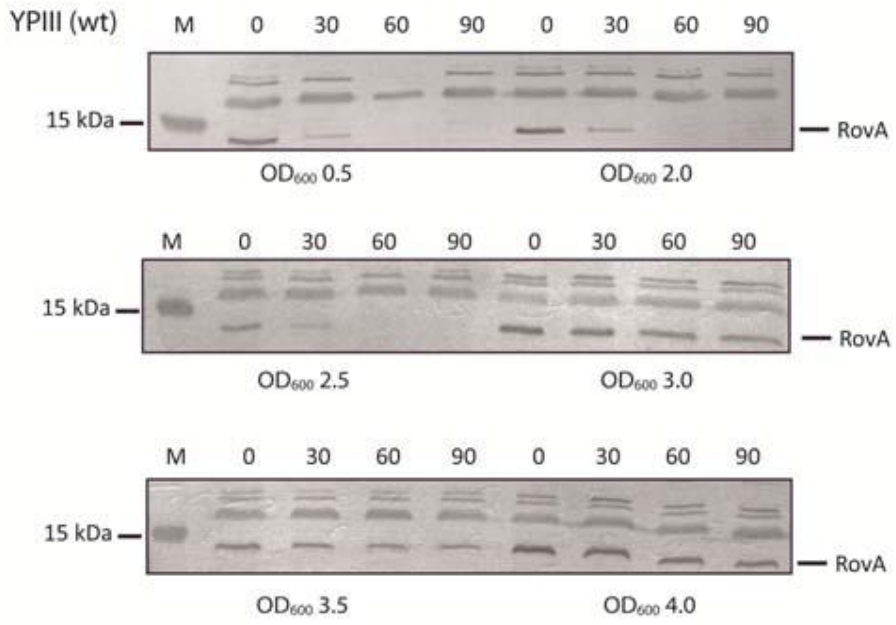


Figure 3-16. Stabilization of RovA occurs in late stationary growth phase. *Y. pseudotuberculosis* strain YPIII was grown to different optical densities (OD_{600}) at 25°C. To stop protein synthesis chloramphenicol (200 µg/ml) was added and cultures were incubated at 37°C for additional 90 min. Samples were taken at indicated time points and analyzed by Western blotting. On the left side a prestained protein marker is added.

The data clearly demonstrate that some stabilization was detectable in mid-stationary growth phase at an OD_{600} of 3 whereas at lower optical densities the protein is com-

pletely degraded 60 min after stop of protein synthesis. At an OD_{600} of 3.5 RovA was only slightly degraded and it remained completely stable in late stationary growth phase at an OD_{600} of 4 (Figure 3-16). This leads to the conclusion that stabilization of RovA occurs during mid- to late-stationary growth phase. Therefore, it seems likely, that a secreted factor which accumulated during stationary phase prevents RovA degradation by the Lon and ClpP proteases.

3.1.11 An unknown factor is released into the medium in stationary growth phase

It has been previously shown that stationary phase growth medium conditioned with specific small molecules was capable to protect exponentially grown cells from oxidative stress or was able to induce competence (Westwater *et al.*, 2005, Magnuson *et al.*, 1994). To test if factor-conditioned YPIII stationary phase medium is able to prevent RovA degradation in exponentially grown cells, bacteria were grown overnight or to exponential growth phase at 25°C. Cultures were centrifuged and the stationary phase supernatant was sterilized and supplemented with 200 µg/ml chloramphenicol. Subsequently, exponentially grown cells were harvested, resuspended in the stationary phase supernatant and incubated at 37°C. As a control, the same assay was performed with unconditioned LB medium. As shown in Figure 3-17, stationary phase supernatant was able to completely prevent RovA degradation in exponentially grown cells, whereas in the control assay RovA was rapidly degraded. In contrast, exponential phase supernatant did not have any effect on RovA stability in stationary grown cells (data not shown). Interestingly, addition of stationary phase supernatant from *E. coli* and *Salmonella* led to similar results (data not shown). Moreover, stationary phase supernatant from cells grown at 37°C was able to stabilize RovA, whereas supernatants of *rovA*$^-$ or *lon*$^-$ cultures had no effect on protein stability. Taken together, these results indicate that the stationary phase factor is released into the growth medium at 25°C and 37°C. It is rapidly taken up and induces stabilization of RovA. However, it is unclear whether the factor is transported into the cell and binds to either RovA or the protease, or if it induces a signal cascade that somehow prevents RovA proteolysis. Furthermore, the same or closely related component is also secreted during stationary phase by *Salmonella* and *E. coli*.

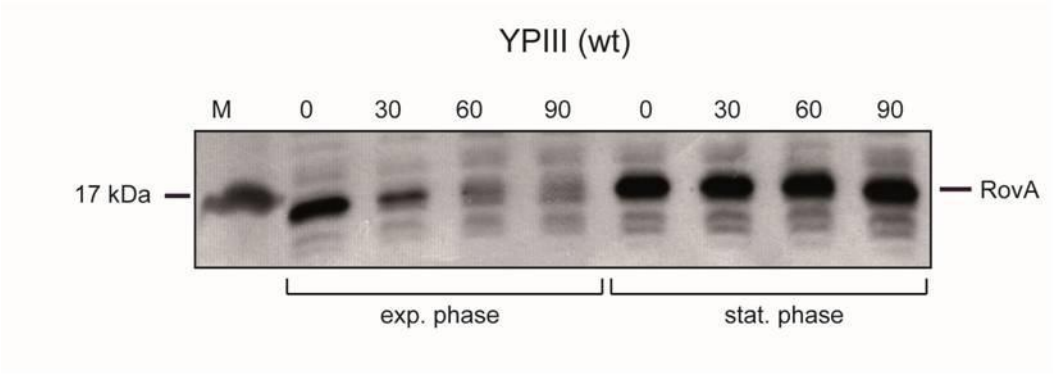


Figure 3-17. Stabilization of RovA in exponentially grown *Y. pseudotuberculosis* YPIII cells by stationary growth phase media supernatant. *Y. pseudotuberculosis* strain YPIII was grown overnight or to exponential growth phase at 25°C. Cells were centrifuged and exponentially grown cells were either resuspended in LB medium or in stationary phase supernatant. To stop protein synthesis chloramphenicol (200 µg/ml) was added. Cultures were incubated at 37°C for additional 90 min and samples were taken at indicated time points. Whole cell extracts with identical numbers of bacteria were prepared and analyzed by Western blotting. On the left side a prestained protein marker is added.

3.1.12 RovA stabilization is strongly controlled by concentration of the secreted compound

Previous experiments addressing RovA degradation during different growth phases indicated that the unknown secreted factor is started to be produced in mid-exponential growth phase. Complete stabilization of RovA was only observed in late stationary growth phase. Therefore, it was assumed that stabilization of RovA is dependent on higher factor concentrations. To confirm this, stationary phase supernatant was diluted 1:1, 3:1 and 7:1 with unconditioned LB medium and stability assays were performed as described previously. All dilutions led to strong destabilization of RovA similarly to the exponential phase control (Figure 3-18). Rapid RovA degradation at a minimal reduction of the factor concentration in the supernatant indicated that the unknown factor is either present in very low amounts in the supernatant or exhibits a very low binding affinity to RovA

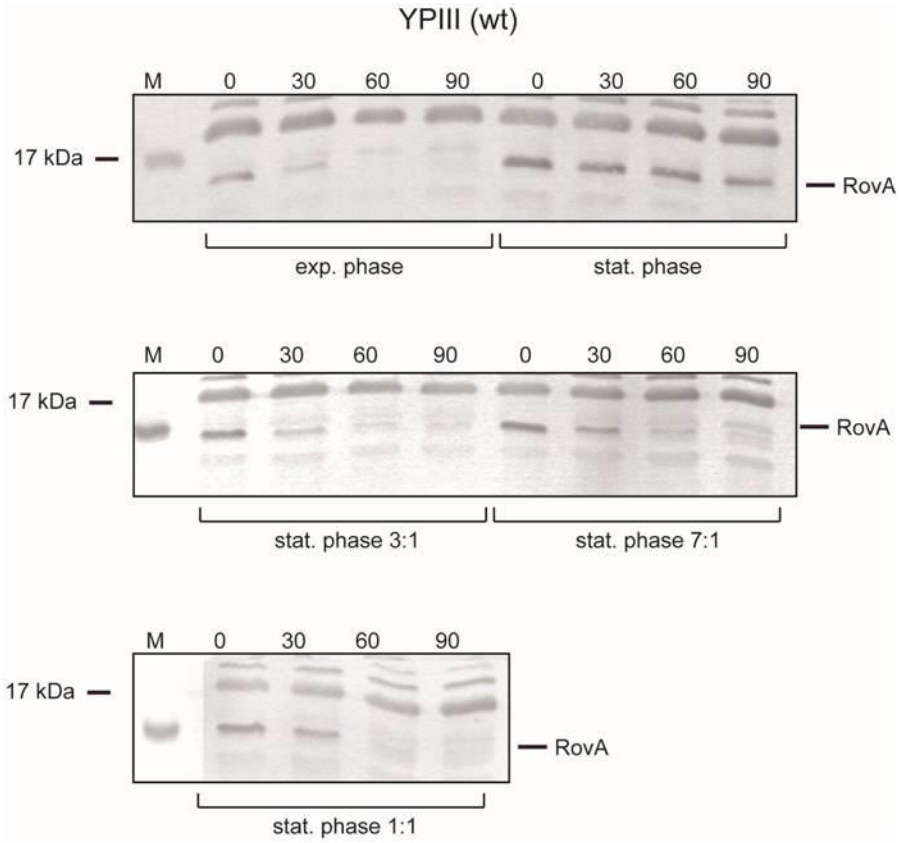


Figure 3-18. Factor-concentration dependent stabilization of RovA. *Y. pseudotuberculosis* strain YPIII was grown overnight or to exponential growth phase at 25°C. Cells were centrifuged and exponentially grown cells were either resuspended in LB medium or in stationary phase supernatant diluted as indicated with fresh LB medium. To stop protein synthesis chloramphenicol (200 µg/ml) was added. Cultures were incubated at 37°C for additional 90 min and samples were taken at indicated time points. Whole cell extracts with identical numbers of bacteria were prepared and analyzed by Western blotting. On the left side a prestained protein marker is added.

3.1.13 The RovA-stabilizing factor is heat stable and Pronase resistant but sensitive to pH

To gain deeper insights into the properties of the RovA-stabilizing factor, experiments were performed addressing factor stability in the stationary phase supernatant. To do so, stationary phase supernatant was cooked and re-cooled several rounds or was heated to 80°C for at least 12 h. Subsequently, *Y. pseudotuberculosis* strain YPIII was grown to exponential growth phase and cells were resuspended in the pre-treated stationary phase supernatant supplemented with 200 µg/ml chloramphenicol. Cultures were incubated at 37°C and samples were taken at the indicated time points. RovA amounts were visualized by Western blotting. As a control, the assay

was performed with fresh LB medium treated in identical fashion. Data clearly demonstrated that boiling of the medium had no effect on RovA stability (Figure 3-19). Heating the medium to 80°C for at least 12 hrs only led to slight degradation of RovA (Figure 3-19), indicating that the unknown factor is quite heat stable. Moreover, stationary phase supernatant was incubated for 2 hrs with Pronase (Roche), a mix of endopeptidases isolated from *Streptomyces griseus* that is degrading native and non-native proteins. Afterwards, assays were performed as described previously. As shown in Figure 3-19 also Pronase treatment had no effect on RovA stability, although the protease mix was able to degrade casein in the stationary phase supernatant (data not shown).

In summary, the RovA-stabilizing factor is heat stable and resistant to Pronase. Therefore, it is quite unlikely that the RovA-stabilizing component is a protein.

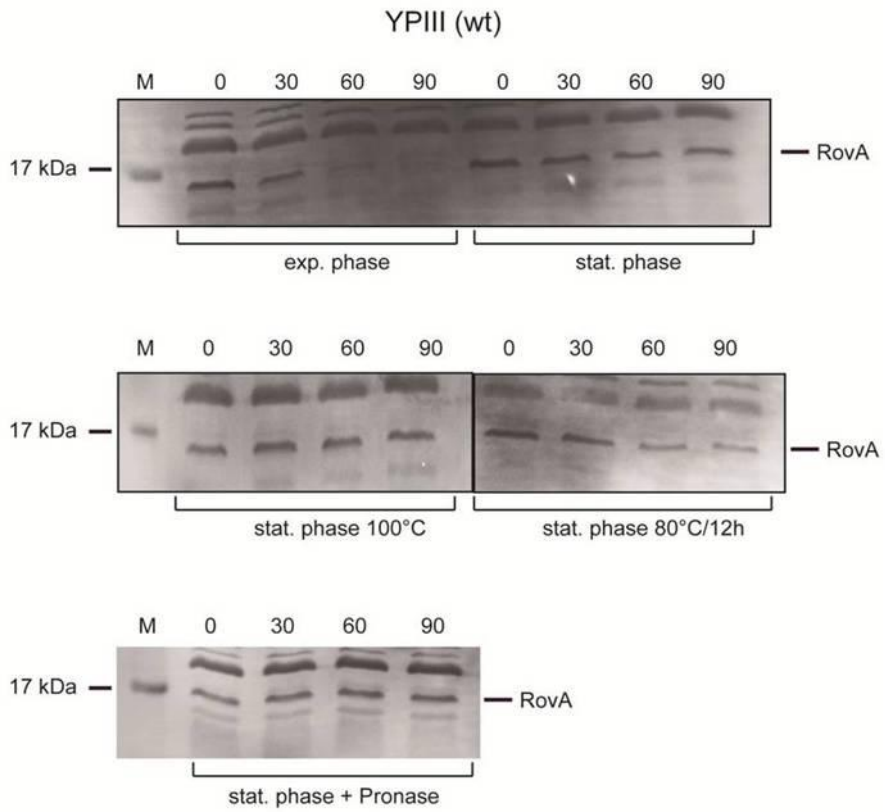


Figure 3-19. Heat and Pronase stability of the RovA-stabilizing factor. *Y. pseudotuberculosis* strain YPIII was grown overnight or to exponential growth phase at 25°C. Cells were centrifuged and exponentially grown cells were either resuspended in LB medium or differently conditioned stationary phase supernatant. To stop protein synthesis chloramphenicol (200 µg/ml) was added. Cultures were incubated at 37°C for additional 90 min. Samples were taken at indicated time points and analyzed by Western blotting. On the left side a prestained protein marker is added.

Additionally to heat- and Pronase stability, factor sensitivity to pH variations was investigated. Stationary phase medium supernatant was found to have a pH of about 8, whereas supernatant from exponentially grown cells has a pH between 6.8 and 7. To exclude that pH alone is responsible for RovA stabilization the pH of stationary phase supernatants was decreased and alternatively the pH of exponential phase supernatant was increased before addition to exponentially grown YPIII cells. As shown in Figure 3-20 lowering the pH of stationary phase supernatants clearly led to destabilization of RovA. Protein degradation was comparable to untreated medium. In contrast, increasing the pH in exponential cell supernatant had no effect on RovA stability. These results demonstrate that growth phase dependent RovA degradation is sensitive to changes in pH, but pH changes alone are not sufficient to promote RovA stabilization. This indicates that the secreted RovA-stabilizing factor might be a molecule that is destroyed or modified (e.g. changes in charge, reaction with other molecules) in acidic environments.

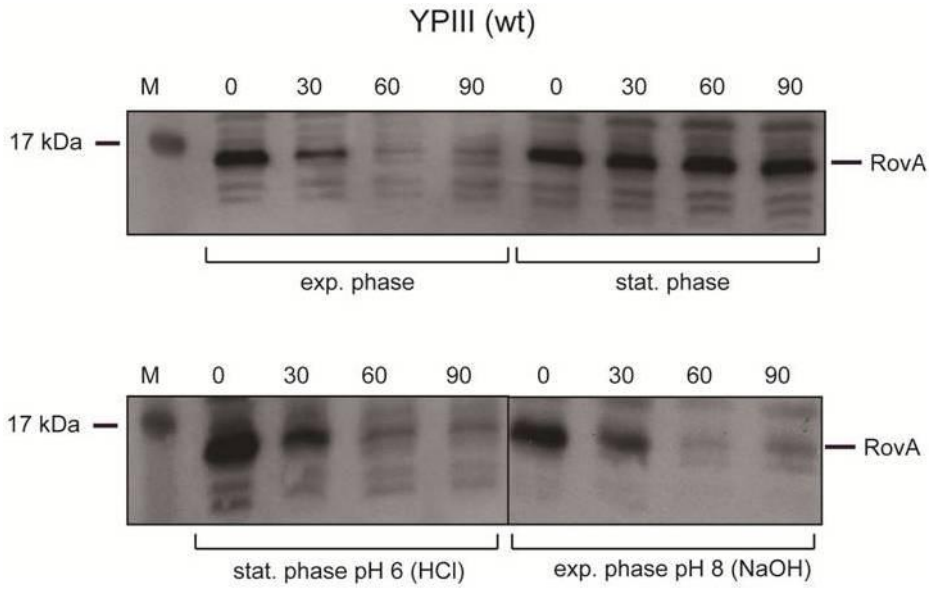


Figure 3-20. pH dependency of RovA stabilization. *Y. pseudotuberculosis* strain YPIII was grown overnight or to exponential growth phase at 25°C. Cells were centrifuged and exponentially grown cells were either resuspended in exponential phase supernatant (pH=8) adjusted with 5 M NaOH or in stationary phase supernatant (pH=6) adjusted with HCl. To stop protein synthesis chloramphenicol (200 µg/ml) was added. Cultures were incubated at 37°C for additional 90 min; samples were taken at the indicated time points and analyzed by Western blotting. On the left side a prestained protein marker is added.

3.1.14 Factor synthesis is dependent on growth medium composition

RovA stability assays demonstrated that the RovA-stabilizing factor accumulates during stationary growth phase and is strongly affected by different environmental conditions. To find out more about the conditions necessary for factor production, the effect of the growth medium composition on RovA stability was tested. To do so, YPIII cells were grown over night at 25°C in the complex media LB and DMEM (Biochrom) and in the minimal media MMA and M9. Subsequently, YPIII cells were grown to exponential growth phase at 25°C and were resuspended in the different stationary phase media supernatants. After stopping protein biosynthesis, cultures were incubated at 37°C, samples were taken at indicated time points and RovA amounts were visualized by Western blotting.

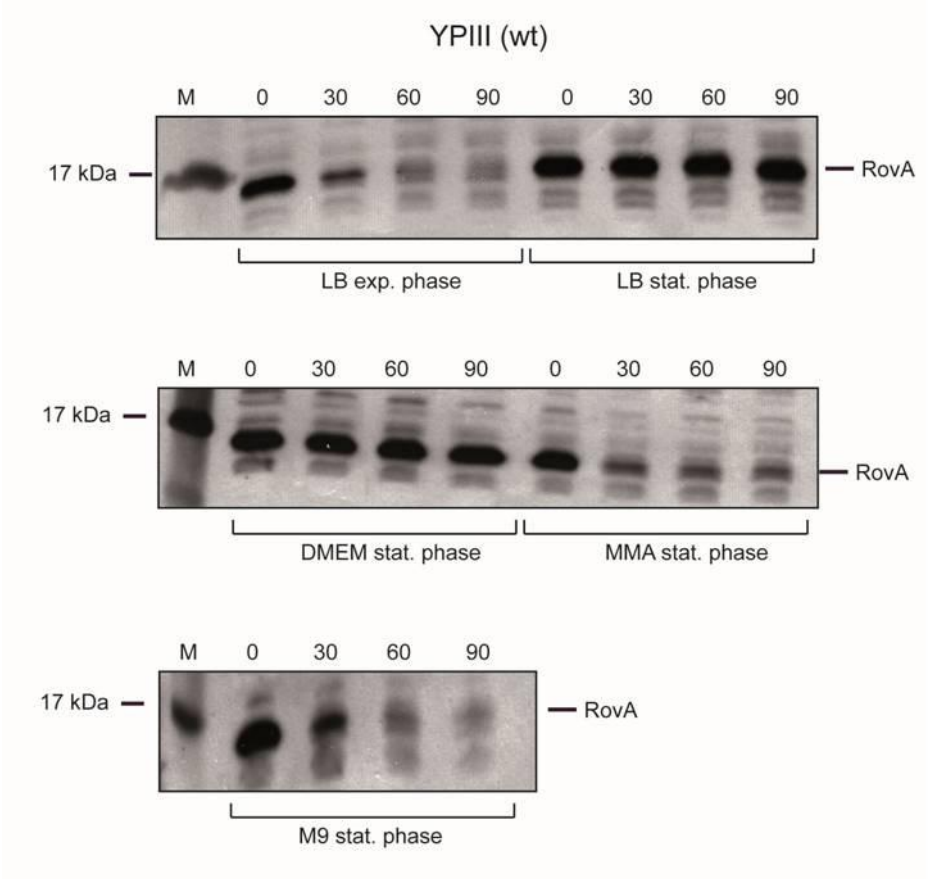


Figure 3-21. Media-dependent stabilization of RovA. *Y. pseudotuberculosis* strain YPIII was grown overnight in LB, DMEM, MMA and M9 medium or to exponential growth phase in LB medium at 25°C. Cells were centrifuged and exponentially grown cells were either resuspended in LB medium or in stationary phase supernatants. To stop protein synthesis chloramphenicol (200 µg/ml) was added. Cultures were incubated at 37°C for additional 90 min; samples were taken at the indicated time points and analyzed by Western blotting. On the left side a prestained protein marker is added.

As shown in Figure 3-21 RovA remains completely stable in complex medium, whereas it is degraded in both minimal media, demonstrating that factor production is not only dependent on growth phase but also on the nutrient content of the growth medium. It is not clear if one or more compounds in the growth medium are needed for RovA stabilization or if the nutrient supply in the environment is serving as a signal to activate a signal transduction cascade within the bacteria. However, based on previous experiments it is most likely that the RovA-stabilizing factor is a metabolic intermediate that is only produced under nutrient rich conditions.

3.1.15 Production of the RovA-stabilizing factor is dependent on CsrA and Crp

Previous studies and microarray analysis demonstrated that the RNA binding protein CsrA and the cAMP receptor protein Crp are strongly involved in adaptation of *Yersinia* metabolism in response to environmental and physiological conditions (AK. Heroven, unpublished). Moreover, both proteins are part of the RovA regulatory network (Figure 1-7). Assuming the RovA-stabilizing factor is a metabolic intermediate, the question was raised if CsrA or Crp is able to affect the production of the secreted factor and therefore have an influence on RovA stability. To address this, a *Y. pseudotuberculosis csrA* mutant (YP53) and a *crp* mutant (YP89) were grown at 25°C overnight. Subsequently, exponentially grown YPIII cells were resuspended in the supernatant of these cultures and degradation assays were performed as described previously. As a control, stationary and exponentially grown *Y. pseudotuberculosis* YPIII wild-type supernatant was tested.

As demonstrated in Figure 3-22, the supernatant of both mutant strains was less able than the wild-type in stabilizing RovA. This leads to the assumption that both proteins control metabolic pathways or transport processes that are essential for factor production, uptake or release. 200 genes were found to be differentially regulated in a *csrA* mutant and 300 genes exhibited a different expression pattern than the wild-type in a *crp* mutant. Most of these genes are involved in carbohydrate and amino acid metabolism (AK. Heroven, unpublished). Due to the global effect of these regulator mutations on various metabolic pathways it was not possible to suggest a specific pathway involved in factor production. Therefore, a different approach was chosen to find the putative stabilizing factor for RovA.

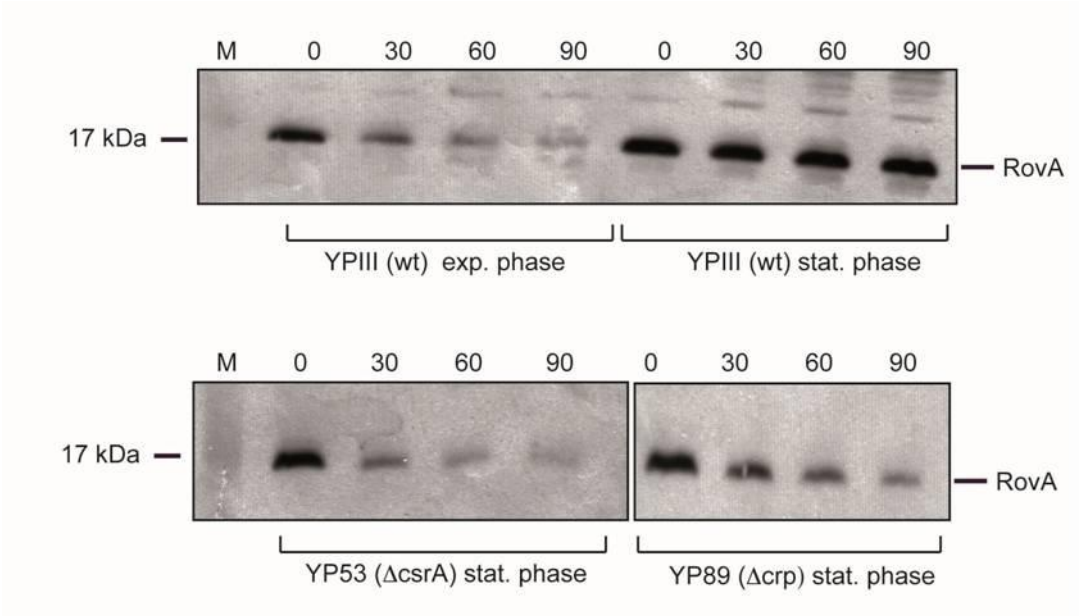


Figure 3-22. Production of the RovA-stabilizing factor is CsrA- and Crp-dependent. *Y. pseudotuberculosis* strains YPIII (wt), YP53 (Δ*csrA*) and YP89 (Δ*crp*) were grown overnight at 25°C. Cells were centrifuged and exponentially grown YPIII cells were resuspended in the stationary phase supernatants. To stop protein synthesis chloramphenicol (200 µg/ml) was added. Cultures were incubated at 37°C for additional 90 min; samples were taken at indicated time points and analyzed by Western blotting. On the left side a prestained protein marker is added.

3.1.16 RovA binds to small aromatic molecules *in vitro*

RovA belongs to the family of MarR-like transcriptional regulators. Many of those MarR family members are known to bind small anionic lipophilic compounds. For example, several putative ligand molecules such as benzoate, 2.4-dinitrophenol, menadion or plumbagin were shown to affect the DNA-binding affinity of MarR *in vivo* and *in vitro* (Alekshun & Levy, 1999a, Alekshun & Levy, 1997, Cohen *et al*., 1993, Martin & Rosner, 1995, Seoane & Levy, 1995). The best characterized interaction of MarR to one of its effector molecules is the binding of the weak aromatic acid salicylate (Alekshun & Levy, 1999a, Alekshun *et al*., 2001, Cohen *et al.*, 1993, Martin & Rosner, 1995). Solving the crystal structure of dimeric RovA revealed a binding pocket in direct proximity of the DNA-binding domain, occupied by an unknown molecule (N. Quade, unpublished). Therefore, it was hypothesized that salicylate might also be an effector molecule for RovA. In fact, it was possible to crystallize the protein bound to salicylate (N. Quade, unpublished). Moreover, *in vivo* stability assays with salicylate-conditioned growth medium revealed that millimolar concentra-

tions of the molecule are able to stabilize the protein (Figure 3-23). Up to now, it is not clear if salicylate is a native ligand of MarR or RovA. Salicylate is formed from chorismate via isochorismate and is a precursor molecule for siderophore synthesis. Iron-uptake in *Yersinia* is mediated by the yersiniabactin system that is highly conserved among the pathogenic *Yersinia* species (Gehring *et al.*, 1998a). Formation of yersiniabactin occurs by a mechanism of non-ribosomal peptide synthesis and involves the genes *irp1*, *irp2*, *ybtU*, *ybtE* and *ybtT* (Carniel, 2001, Gehring *et al.*, 1998b). Involvement of iron in regulation of RovA stability would fit to previous results, demonstrating that addition of iron to the growth medium activates *rovA* expression via CsrC (S. Nordlohne, unpublished data). However, comparison of *Yersinia* genomes revealed that Y. p*seudotuberculosis* strain YPIII lacks the genes responsible for yersiniabactin synthesis. Therefore, it is possible that one or more salicylate-related molecules with a similar or even higher binding affinity might be responsible for RovA stabilization in stationary growth phase.

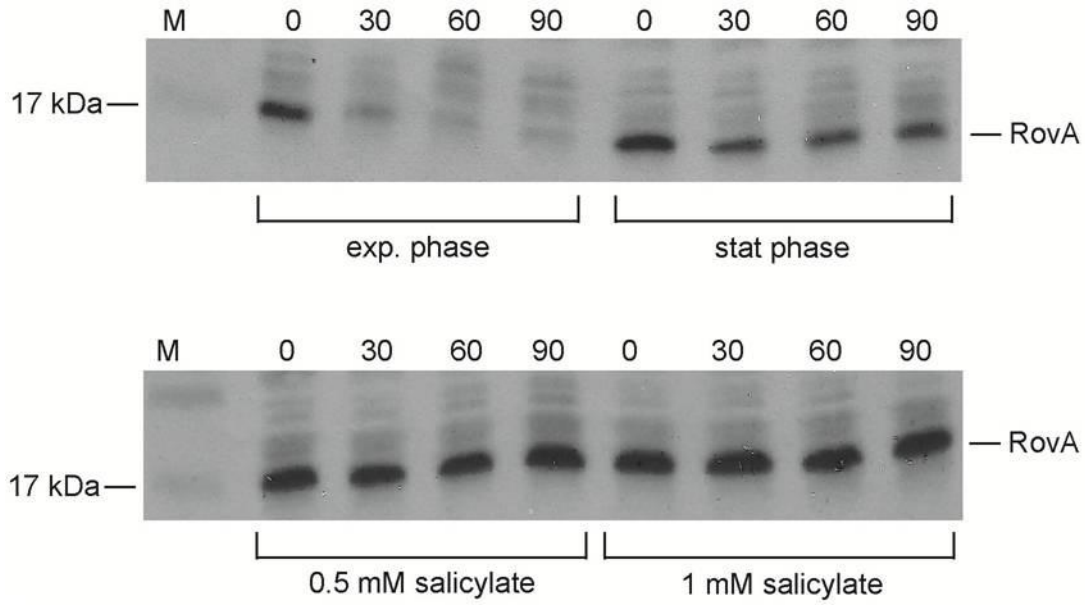


Figure 3-23. RovA stabilization by salicylate. *Y. pseudotuberculosis* strain YPIII was grown overnight or to exponential growth phase in LB medium at 25°C. Cells were centrifuged and exponentially grown cells were either resuspended in LB medium, LB medium supplied with 0.5 or 1 mM salicylate or in stationary phase supernatant. To stop protein synthesis chloramphenicol (200 µg/ml) was added. Cultures were incubated at 37°C for additional 90 min; samples were taken at the indicated time points and analyzed by Western blotting. On the left side a prestained protein marker is added.

Recently, Chubiz and Rao (Chubiz & Rao, 2010) could show that the metabolic intermediates 2,3-dihydroxybenzoate, anthranilate and 4-hydroxybenzoate involved in enterobactin, tryptophane and ubiquitin biosynthesis respectively are able to activate the *marRAB* operon in *E. coli*. However, only 2,3-dihydroxybenzoate was able to affect MarR activity by direct binding to the protein (Chubiz & Rao, 2010). Accordingly, numerous potential effector molecules structurally similar to salicylate were investigated with respect to RovA binding *in vitro*. Most of the chosen metabolic intermediates are involved in the superpathway of chorismate and are listed in Table 7. 2,3-dihydroxybenzoate is an intermediate of benzoate degradation and is involved in Enterobactin synthesis in *E. coli* and *Salmonella* (Raymond *et al.*, 2003). 4-hydroxybenzoate is an intermediate in ubiquinone biosynthesis whereas anthranilate and 4-aminobenzoate are intermediates in tryptophane biosynthesis and tetrahydrofolate biosynthesis, respectively. The structurally different intermediate uric acid is involved in nucleic acid metabolism but was found to be the native ligand for the MarR family regulator PecS in *Erwinia* and HucR in *Deionococcus radiodurans* (Perera & Grove, 2010, Perera *et al.*, 2009). Microarray analysis of the *Y. pseudotuberculosis* YPIII wild-type and a *rovA* mutant revealed an activating effect of RovA on the urease-coding operon *uraABC* (AK. Heroven, unpublished data). Therefore, it might be possible that uric acid is also a ligand for RovA. Phenylpyruvate, phenylalanine and cinnamate are intermediates or end products of the phenylalanine and tryptophane biosynthetic pathways.

To test whether RovA directly binds to the selected molecules, ThermoFluor analysis was performed with 2 mg/ml purified RovA and increasing amounts of putative ligand molecule. Protein T_m was plotted against the ligand concentration and the dissociation constant was calculated. An example of the obtained binding curves for salicylate and uric acid is illustrated in Figure 3-24. Whereas uric acid was not able to bind to RovA, a K_d of 2.24 mM could be determined for salicylate. K_d values of other metabolic intermediates are listed in Table 7.

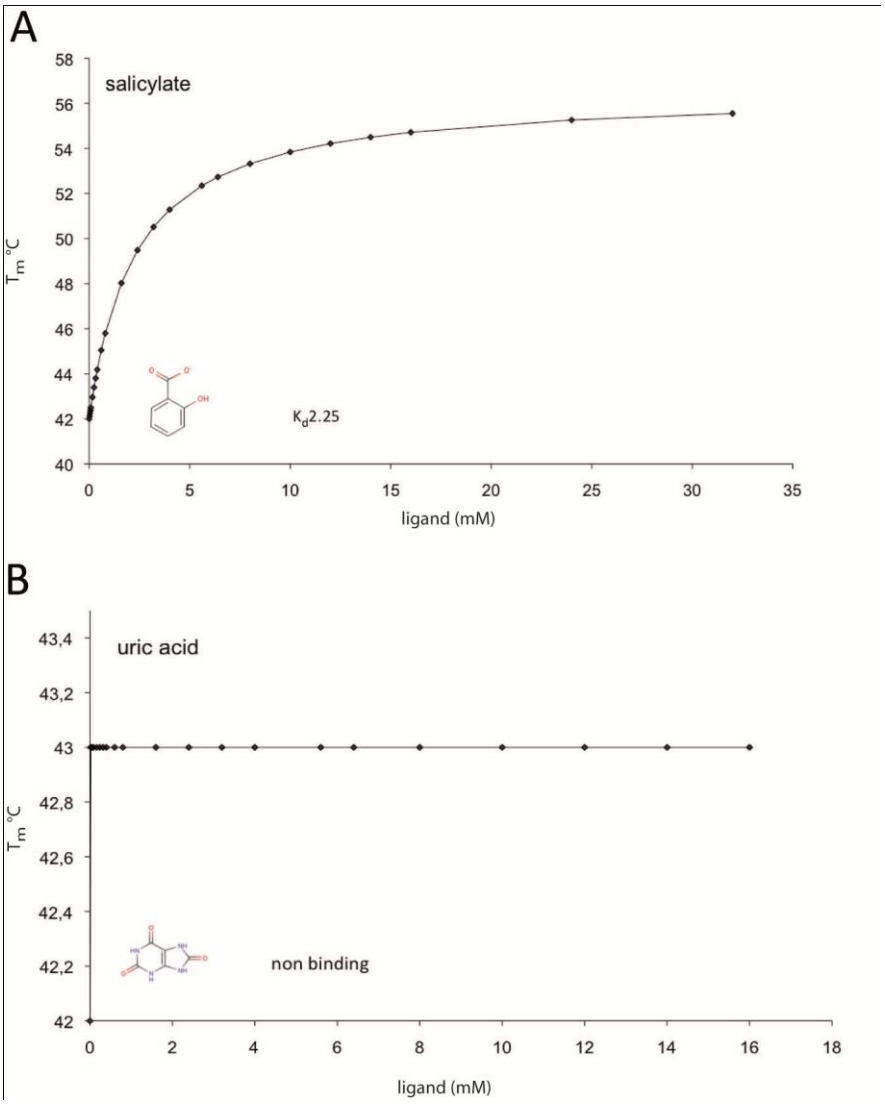
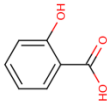
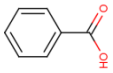
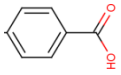
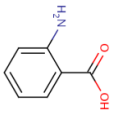
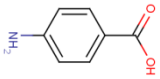
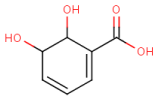
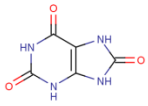
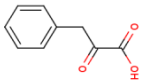
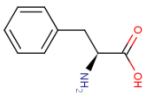
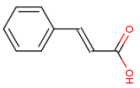


Figure 3-24. Analysis of RovA-ligand interactions. The binding affinity of salicylate **(A)** and uric acid **(B)** to RovA was determined by ThermoFluor analysis. 2 mg/ml of protein was mixed with different ligand concentrations and the effect of ligand binding on protein T_m was measured. The temperature was increased from 10°C to 90°C in 0.5°C increments and ligand-binding was recorded with an excitation wavelength of 495 nm and an emission wavelength of 610 nm. The K_d value was defined as the ligand concentration necessary for half occupying the RovA binding site and was calculated as described previously (2.5.3.8).

Table 7. Binding affinities of putative RovA ligand molecules. K_d values were determined by using ThermoFluor analysis.

compound	structure	K_d [mM]
salicylate		2.25
4-hydroxybenzoate		2.75
benzoate		2.93
anthranilate		3.92
4-aminobenzoate		5.86
2,3-dihydroxybenzoate		2.33
uric acid		no binding
phenylpyruvate		3.52
phenylalanine		no binding
cinnamate		1.27

2,3-dihydroxybenzoate is binding to RovA with almost the same affinity (2.33 mM) as salicylate. Differently, benzoate, lacking one hydroxyl group at position C_2, and 4-hydroxybenzoate harbouring the hydroxyl group at position C_4, were binding with slightly lower affinities (2.93 mM and 2.75 mM). In contrast, anthranilate and 4-aminobenzoate, both harbouring an amine group, possess lower binding affinities (3.92 mM and 5.86 mM). Cinnamate had the highest binding affinity and was found to bind with a K_d of 1.27 mM. Interestingly, cinnamate binds 2-3-fold better than phenylpyruvate (2.52 mM), although the molecule contains just one additional hy-

droxyl group. An exchange of this hydroxyl group to an amine group completely abolished RovA-binding. Stabilization of RovA by all putative ligands was also confirmed by protein degradation assays *in* vivo (C. Mendonca, unpublished data).
Taken together, these results indicate that the position of the hydroxyl group on the benzyl ring has a strong influence on effector binding by RovA. Moreover, the presence of an amine group seems to strongly reduce or even completely abolish binding to RovA.

3.2 Heterogeneous *rovA* expression in *Y. pseudotuberculosis*

The ability of a bacterium to survive under different environmental conditions strongly depends on its gene expression pattern. Bacterial populations grown in homogenous environments have always been regarded as identical in terms of their gene expression patterns and the response to changing environmental conditions was considered to happen in a more-or-less uniform manner (Dubnau & Losick, 2006). However, with the advent of new techniques allowing to measure gene expression in single cells, it became evident that bacteria may possess two or more gene expression states within the same population (Graumann, 2006). This population heterogeneity was observed in various bacterial systems and can either result from genetic rearrangements and modifications (Hallet, 2001, Low *et al.*, 2001) or by a mechanism based on positive or double negative feedback loops within a gene regulatory network (Smits *et al.*, 2006). This type of population heterogeneity is referred to as bistability and is characterized by the presence of two alternative stable gene expression states (“ON” and “OFF”) in one population, which can reversibly switch between each other. Modelling of synthetic gene regulatory circuits demonstrated that bistability is generated by specific feedback in combination with a nonlinear response within a regulatory network (Ferrell, 2002, Hasty *et al.*, 2002, Thomas, 1998). Nonlinearity may occur from cooperative binding of a transcriptional regulator to the DNA, changes in protein concentrations due to regulated proteolysis or when multimerization is required for regulator activity (Smits *et al*., 2006, Buchler *et al.*, 2005).

The regulation of *rovA* expression occurs via a complex regulatory network (Figure 1-7). One interesting feature of this network is the autoregulatory control of *rovA* expression by positive and negative feedback. RovA is able to cooperatively activate its

own transcription upon binding to an extended AT-rich sequence in the upstream region of promoter P2, resulting in a positive feedback loop (Heroven *et al*., 2004). At higher concentrations, RovA binds to a low affinity site upstream of the P1 promoter and represses its own transcription (Heroven *et al*., 2004). Moreover, in this study it was demonstrated that RovA levels are modulated by temperature-dependent degradation and DNA-binding. These regulatory properties of *rovA* expression perfectly fulfil the requirements for bistability. In fact, mathematical modelling of *rovA* expression predicted bistable expression, mediated by positive feedback and temperature-dependent degradation and DNA-binding (Klein, 2010).

In order to validate this modelling approach, the temperature dependent expression of *rovA* in an isogenic population was monitored using flow cytometry and fluorescence microscopy.

To this purpose, plasmid pKH70 was constructed, harboring the *gfp* gene under the control of the *rovA* promoter. Since the GFP protein exhibits a half-life up to 24 hrs an unstable variant of GFP (GFP-LVA) (Andersen *et al.*, 1998) was chosen to directly monitor the temperature induced changes in RovA levels.

Plasmid pKH70 was introduced into *Y. pseudotuberculosis* strain YPIII and cells were grown overnight at different temperatures (30°C to 34°C at 1°C increments). The cultures were diluted 50-fold with fresh medium and grown for additional 3 hrs at the same temperatures. Samples were collected and prepared for FACS analysis and fluorescence microscopy. *Y. pseudotuberculosis* YPIII wild-type cells and YPIII pFU95 cells harboring the *gfp* gene under control of the constitutively expressed *gapA* promoter of the glycerinaldehyde-dehydrogenase gene (*gapA*) were grown at 37°C and 25°C, respectively, and were used as negative and positive controls.

As shown in Figure 3-25A and B *rovA* expression is completely repressed at 34°C whereas almost all cells express the gene at 30°C. In contrast, flow cytometry analysis clearly revealed the presence of two distinct populations at 32°C: one population that is expressing *rovA* (“ON”) and one population that is “OFF”, with a slight predominance of the expressing cells. Only very small subpopulations in the on “ON” or “OFF” state could be observed at 31°C and 33°C. These results are in agreement with Western blot analysis of YPIII pKH70 (Figure 3-25C). Levels of chromosomally encoded RovA are high at moderate temperatures (30°C) whereas at higher temperatures (34°C), under RovA non-inducing conditions, protein levels are low. At 32°C,

when *rovA* is heterogeneously expressed, protein levels are similar to those at moderate temperatures, indicating that not only the amount of RovA but also protein conformation and consequently, the DNA-binding capacity play a role in heterogeneous *rovA* expression.

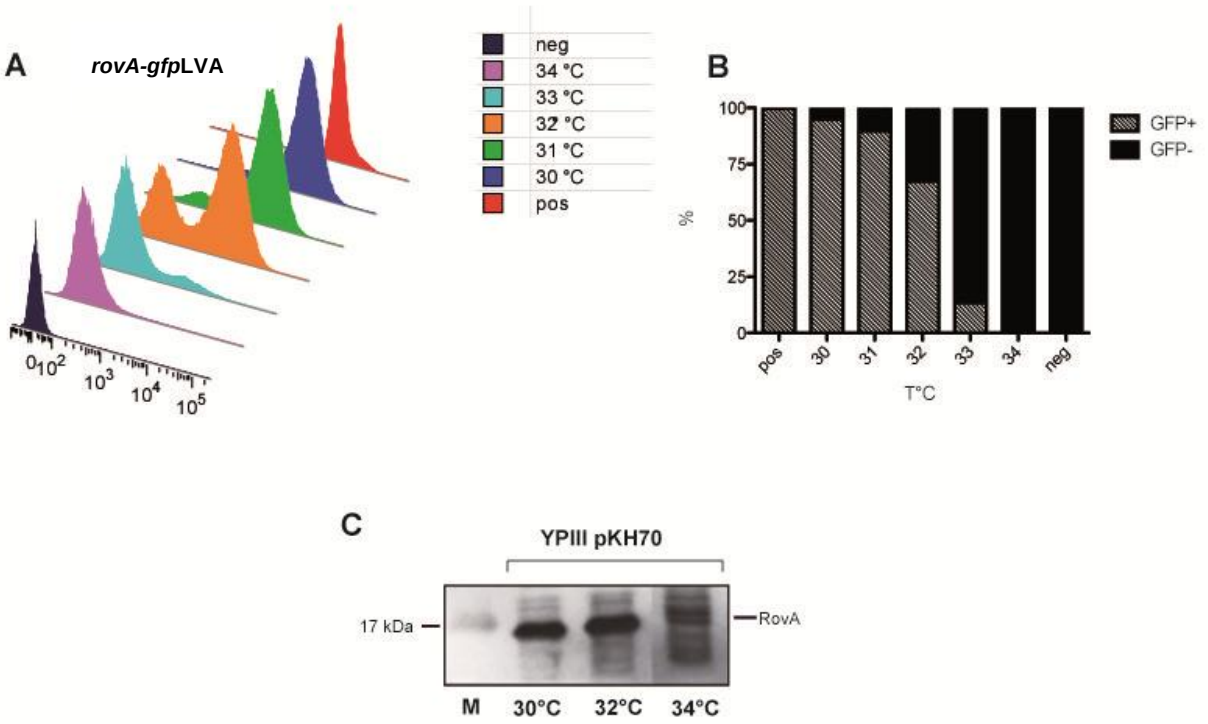


Figure 3-25. Heterogeneous expression of a *rovA-gfpLVA* fusion in *Y. pseudotuberculosis* YPIII. YPIII cells harboring the plasmid pKH70 (*rovA-gfp*LVA) were grown to exponential growth phase at different temperatures and samples were prepared for FACS analysis **(A)**. Colored peaks represent detection of GFP at corresponding temperatures. The x-axis shows fluorescence intensity in arbitrary units given in a logarithmic scale. As positive (pos) and negative (neg) controls YPIII cell harboring the plasmid pFU95 (*gapA-gfp*) were grown at 25°C (pos) and 37°C (neg). **(B)** Quantitative determination of GFP^+ (grey bars) and GFP^- (black bars) cells at indicated temperatures measured by FACS analysis. **(C)** Western blot analysis visualizing amounts of chromosomally encoded RovA in YPIII pKH70 at 30°C, 32°C and 34°C.

Additionally, expression of the *rovA-gfp*LVA fusion was investigated by fluorescence microscopy, confirming the results obtained from FACS analysis. Representative images of cells grown in a temperature range from 30°C to 34°C are presented in Figure 3-26. At moderate temperatures the reporter fusion is expressed in all cells, whe-

reas it is completely switched off at 34°C. In contrast, at 32°C almost 70% of the cells express *rovA* whereas around 30% do not.

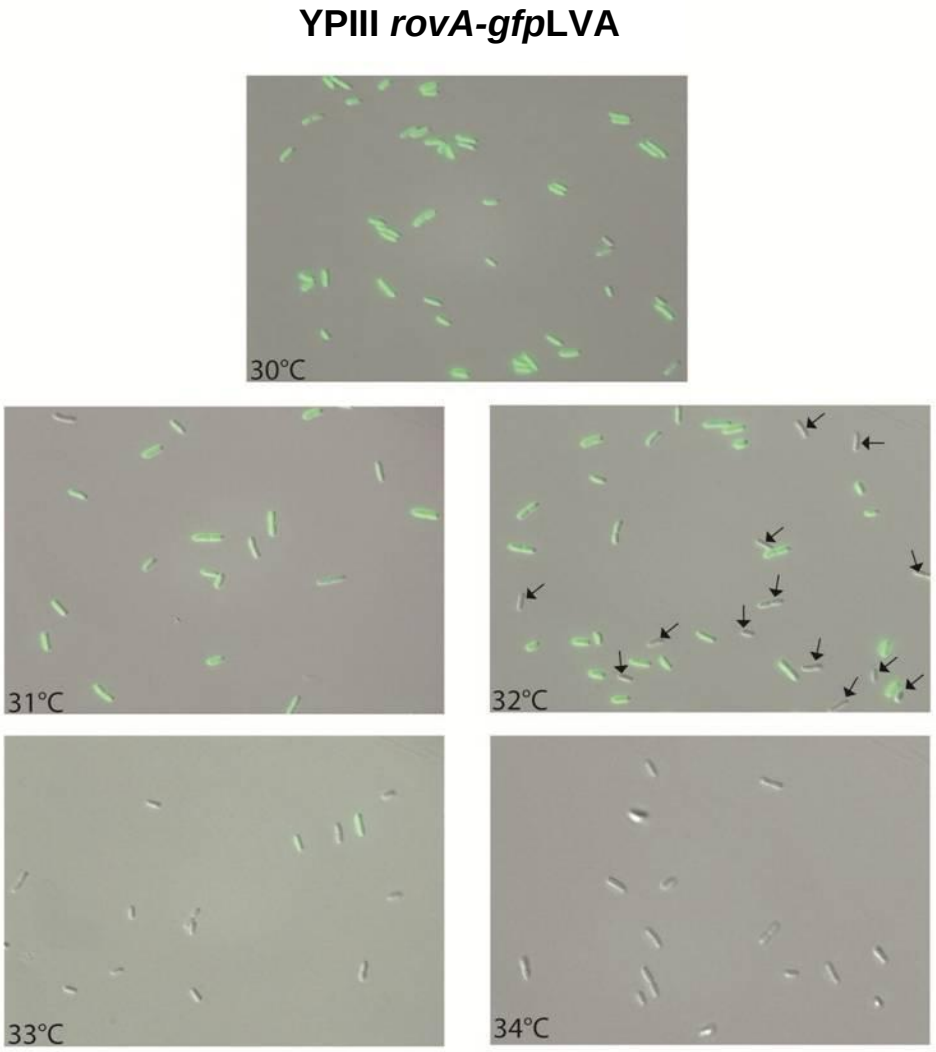


Figure 3-26. Fluorescence microscopy of *Y. pseudotuberculosis* YPIII *rovA-gfp*LVA. YPIII cells harboring the plasmid pKH70 (*rovA-gfp*LVA) were grown to exponential growth phase at different temperatures and samples were prepared for fluorescence microscopy as described previously. Representative micrographs of green coloured fluorescence images were overlaid with the corresponding DIC image. Arrows indicate the non-GFP expressing cells.

The unstable GFP-LVA variant used in this study was constructed by adding a degradation-tag based on the sequence of the SsrA-tag. The SsrA-tag is known to be a substrate for Clp and Lon proteases (Andersen *et al.*, 1998, Lies & Maurizi, 2008). Previous results in this study demonstrated that synthesis of the Lon protease is de-

pendent on temperature. Higher amounts of Lon were detected in YPIII cell extracts grown at 37°C, whereas protease synthesis significantly decreased in cells grown at 25°C. To exclude that stability of the GFP-LVA protein is affected by temperature, GFP degradation assays were performed at 37°C, 34°C, 32°C, 30°C and 25°C. Degradation was quantified and is presented in Figure S2. These data demonstrate that GFP-LVA stability in general depends on temperature. However, within the temperature range when a homogeneous population switches to a heterogeneous population and *vice versa* (30° to 32°C) GFP-LVA stability seems to be identical. This indicates that culture heterogeneity is not a result of temperature-dependent degradation of the reporter protein but exclusively results from the bistable expression of *rovA*.

4 Discussion

4.1 Temperature and growth phase dependent regulation of RovA

During the life cycle of a bacterial pathogen, adaptation of virulence gene expression to environmental conditions is one of the most important mechanisms for pathogenesis, as it is crucial for efficient colonization and infection of the host. Shifting between the external environment and the mammalian host means being exposed to rapidly changing conditions and temperature is believed to be an important signal for many bacteria to start adjusting their virulence gene expression program (Konkel & Tilly, 2000). Pathogenic *Yersiniae*, in which temperature is known to be the major factor in modulating virulence gene expression (Straley *et al*., 1993) are a useful model to study mechanisms for temperature dependent gene regulation. Commonly, temperature-dependent gene expression is regulated on a transcriptional or post-transcriptional level, by DNA-supercoiling or changes in mRNA conformation, but also post-translational mechanisms have been found (Hurme & Rhen, 1998). A post-transcriptional regulatory mechanism for example was shown for LcrF in *Y. pestis*. At moderate temperatures, the mRNA of LcrF forms a loop structure that blocks the Shine-Dalgarno region and therefore prevents binding of the RNAP. At 37°C the structure melts and the *lcrF*-mRNA is translated (Hoe & Goguen, 1993). A similar thermoswitch-like mechanism was shown for LcrF/VirF in *Y. pseudotuberculosis* (K. Boehme, R. Steinmann, unpublished data). An example of transcriptional regulation was discovered for the virulence regulator YmoA of *Y. enterocolitica*. The protein represses *virF/lcrF* expression at moderate temperatures by modulation of the DNA-topology (Cornelis, 1993). The global virulence regulator RovA in *Y. pseudotuberculosis* was shown to be strongly regulated by temperature and growth phase. However, none of the known factors in the RovA regulatory network (Figure 1-7) seems to be responsible for these effects.

In this study a post-translational mechanism for temperature and growth phase dependent regulation of RovA was demonstrated.

4.1.1 RovA – a temperature sensing protein

Experimental data of this study revealed that the MarR-like global virulence regulator RovA of *Y. pseudotuberculosis* acts as an intrinsic protein thermometer that controls its DNA-binding ability by temperature induced alterations of the protein structure. A temperature shift from 25°C to 37°C led to a significant loss of cooperative DNA-binding capacity to the *rovA* and *inv* regulatory region, due to partial defolding of the protein. Predicted α-helical structures were lost, whereas the content of β-sheets increased at higher temperatures. This alteration of secondary structure elements upon temperature changes was found to be completely reversible and seems to be important for the regulatory function of RovA. The conformational changes from an active to an inactive form in response to temperature would be a clever strategy to quickly adjust to environmental conditions inside and outside the host.

In this study, it was shown for the first time that a MarR-type regulator is able to sense temperature. There are only two other bacterial regulators described that are able to directly response to temperature by alteration of protein conformation and those belong to a different class of regulatory proteins. The transcriptional regulator TlpA of *Salmonella enterica* serovar Typhimurium is a dimeric autoregulatory repressor that was shown to use its folding equilibrium to regulate its DNA-binding activity (Hurme *et al*., 1997). The protein contains an N-terminal DNA-binding region and a long left-handed superhelical coil-coil domain. Coiled-coils are formed by sequences containing heptad repeats. The heptad-containing α-helices from each subunit wind around each other and pack their side chains in a “knobs-into-holes” manner, producing the coiled-coiled dimeric structure (Hurme *et al*., 1997). Coiled-coil structures are versatile and very flexible in mediating protein-protein interactions and can be described by a temperature and concentration dependent equilibrium between unfolded monomers and folded oligomers. In TlpA this equilibrium acts as a temperature sensor. Temperature upshifts to 43°C lead to reversible changes in equilibrium that favors the unfolded monomeric form, whereas moderate temperatures lead to formation of the DNA-binding dimeric coiled-coiled structure (Hurme & Rhen, 1998). The function of TlpA is not yet fully understood but the previously postulated involvement in *Salmonella* virulence could not be supported (Gal-Mor *et al.*, 2006). The second known thermo-sensing protein is the repressor RheA, a regulator of the heat shock protein HSP18 in *Streptomyces albus*. RheA is autoregulated and undergoes revers-

ible temperature-induced conformational changes. These lead to alterations in its DNA-binding capacity and either activate (< 30°C) or inactivate (> 40°C) the protein. Also for RheA an α-helical coiled-coiled motif was predicted. Furthermore, the protein contains a leucine-zipper like dimerization motif (Servant *et al*., 2000).

The RovA structure is significantly different from the TlpA and RheA structures. The internal region contains a winged helix-turn-helix motif that binds to the DNA. The first N-terminal and the last two C-terminal α-helices mediate the formation of an extensive and well-packed dimer interface. Because of this type of dimer formation, it is very unlikely that RovA thermo-sensing is based on a TlpA-like monomer-to-dimer conversion. Furthermore, no evidence suggesting dissociation of the dimers into monomers at 37°C was found. In contrast, the melting point for RovA was determined to be 44.5°C and stable RovA dimers could still be detected after heating the protein to 95°C in SDS sample buffer (H. Tran-Winkler, unpublished data).

Recently, the DNA-bound RovA crystal structure has been solved (N. Quade, unpublished data). So far, it is not clear which domains of RovA are affected by temperature. The homologue of RovA in *E. coli* is the virulence regulator SlyA. Investigation of SlyA synthesis at 25 and 37°C indicated that the protein is not sensitive to temperature (A. Wagner, unpublished data). Moreover, *in vivo* degradation assays demonstrated that SlyA is not affected by temperature-induced proteolysis (C. Mendonca, unpublished data). Structural comparison of RovA and SlyA suggests that the temperature sensitive regions are harbored in the α_5 and α_6 helices creating the surface of each dimer. Whereas the N-termini and the DNA-binding domain are highly conserved, the two C-terminal helices differ strongly in their protein sequence. Changing the RovA amino acid sequence to the SlyA sequence, thereby abolishing the RovA thermo-sensing properties could help to better understand the mechanism of temperature dependent RovA regulation. So far, mutational analysis is in progress (N. Quade, C. Mendonca, unpublished data).

4.1.2 RovA as a target of Lon and Clp protease

In addition to the temperature induced conformational changes and the resulting loss of DNA-binding capacity, this study further demonstrated that RovA is also subject to temperature induced regulatory proteolysis. Alterations of the RovA level at different

growth conditions indicated that RovA stability is affected by temperature and growth phase. It could be shown that the RovA protein is stable at 25°C in stationary growth phase, but becomes highly unstable at 37°C in exponential growth phase. Degradation under these conditions is mainly mediated by the Lon protease but also the Clp proteases seem to have a small impact on RovA stability.

The Lon and Clp proteases belong to the superfamily of cytosolic ATP-dependent AAA+ ATPases (ATPases associated with a variety of cellular activities) (Gottesman, 2003). The Lon protease plays a major role in protein quality control. It forms a ring-shaped hexamer with identical subunits and a central cavity forming the proteolytic chamber. Each subunit consists of an N-terminal domain, involved in substrate recognition and binding, a central ATPase domain, containing an ATP binding Walker motif and a C-terminal domain, harboring the proteolytic active site formed by a serine-lysine catalytic dyad (Tsilibaris *et al.*, 2006). The Clp protein is a two component protease consisting of two heptameric ClpP rings and two hexameric ring-shaped ATPase domains (e.g. ClpA or ClpX in Gram-negative bacteria). The ClpP rings form the proteolytic chamber with the active site of a serine protease, while the ATPase domains determine substrate specificity (Yu & Houry, 2007). Both families of proteases share the same properties: (i) the proteolytic site and the ATPase activity is encoded on separate domains or subunits, (ii) the proteases form a multisubunit complex and access to the proteolytic site is gated or mediated by the ATPase domain (self-compartmentalizing proteases), (iii) ATP hydrolysis is required to unfold the substrate, translocate it into the proteolytic chamber and subsequently degrade it into peptide products of approximately 10 to 15 amino acids (Gottesman, 2003).

Proteases play an important role in protein quality control by removing improperly folded or damaged proteins from the cytoplasm and restoring amino acid pools. It has become more and more evident that the directed proteolysis of certain proteins is a crucial instrument of physiological regulation in bacteria (Gottesman, 2003). Lon and Clp proteases degrade naturally unstable proteins that are involved in a great variety of biological processes, e.g. SulA that regulates cell division in *E. coli* (Ishii & Amano, 2001), RscA, a transcriptional activator of capsule synthesis (Torres-Cabassa & Gottesman, 1987), CcdA, the antitoxin of *ccd* post-segregational killing system carried by the F plasmid (Van Melderen *et al.*, 1996) RhlR of *Pseudomonas aeruginosa*, that is involved in quorum sensing (Takaya *et al.*, 2008) or UmuD, involved in repair of damaged DNA (Frank *et al.*, 1996).

Regulated proteolysis was also found to play a role in bacterial virulence. The Lon protease for example is involved in transcriptional regulation of the T3SS in different pathogenic bacteria (Butler *et al.*, 2006). In *Salmonella enterica* serovar Typhimurium Lon degrades the regulator proteins HilC and HilD that regulate HilA, a major regulator of the T3SS that is required for invasion of epithelial cells (Takaya *et al.*, 2005, Zieg *et al.*, 1977, Boddicker & Jones, 2004, Takaya *et al*., 2008, Webb *et al.*, 1999, Linehan *et al*., 2005). Also the T3SS of *Y. pestis* is regulated by Lon and Clp proteases. Expression of the T3SS genes requires the transcriptional activator LcrF that is repressed by the small histone-like protein YmoA. YmoA has been shown to be degraded by the Lon and ClpXP proteases at 37°C but remains stable at moderate temperatures, similarly to RovA as shown in this study (Jackson *et al*., 2004). This is a remarkable connection because the AAA+ proteases seem to oppositely modulate the regulation of two major virulence regulators in *Yersinia* that are involved in expression of early (invasin) and late virulence factors (YadA, Yops). This effect was also observed in *Salmonella enterica* serovar Typhimurium. Besides regulation of HilA by HilC and HilD degradation, Lon is important for systemic infection of mice (Takaya *et al.*, 2005), suggesting a role in upregulation of SPI-2 genes involved in the latter stages of infection (Hensel, 2000). Opposite control of major virulence factors in pathogenic bacteria leads to the assumption that proteases could play a key-role in adaptation to virulence processes during the course of an infection.

The mechanism by which Lon and Clp recognize their substrates for regulated proteolysis is not fully understood. Although Lon has been found to recognize certain key amino acids or domains rich in aromatic residues (so-called degrons), mainly located at the N- or C-terminal end of the target protein, no consensus motif has yet been identified. For the UmuD mutagenesis protein of *E. coli* the recognition site for the Lon protease was found at the N-terminus between amino acid 15 and 18 (Gonzalez *et al.*, 1998), whereas for efficient degradation of *E. coli* SoxS the first 17 amino acids are necessary (Shah & Wolf, 2006b). Degradation of SulA requires the C-terminal histidine (Ishii & Amano, 2001) but also cleavage in the internal region of the protein has been demonstrated (Nishii *et al.*, 2002). For the heme-binding protein HemA of *Salmonella typhimurium*, a N-terminal recognition sequence was postulated and addition of two positively charged amino acids to this region lead to complete stabilization of the protein (Wang *et al.*, 1999b). HemA is rapidly degraded in normal-

ly growing cells but is stabilized in heme-limited cells. Therefore it was proposed that binding of heme to HemA could modify its conformation and lead to the exposure of the tag sequence recognized by the Lon and ClpP protease (Wang *et al.*, 1999a).
Another mechanism of regulated proteolysis that enables substrate quality control is the addition of an SsrA-tag to the C-terminus of a protein for which biosynthesis cannot be normally completed. This addition is directly mediated by the ribosome and leads to rapid degradation of tagged, incomplete polypeptides by Lon and Clp proteases (Lies & Maurizi, 2008, Choy *et al.*, 2007, Baker & Sauer, 2006). Moreover, Gur and Sauer (Gur & Sauer, 2009) demonstrated that degrons are also able to act as regulatory elements by influencing the activity of the Lon protease. Allosteric binding of different degrons leads to alteration of protease conformation and therefore to different proteolytic activities. This mechanism allows the protease to operate in either fast or slow proteolytic mode according to specific physiological needs (Gur & Sauer, 2009).

Experiments of the present study demonstrated that fusion of the N-terminal 96 amino acids of RovA to a normally stable protein leads to degradation by the Lon protease, whereas fusion of the first 42 amino acids does not. These amino acids are located in the direct vicinity of the DNA-binding domain. Also introduction of a mutation minimizing RovA DNA-binding function made the protein more susceptible to proteolysis. In contrast, the presence of a multi-copy plasmid harboring high affinity RovA-binding sites reduced degradation of the protein. These results suggest not only that amino acid residues located inside the helix-turn-helix DNA-binding domain include the signal or recognition site necessary for Lon degradation, but they also indicate that substrate recognition and degradation by the Lon protease could be in direct correlation with the DNA-binding activity of RovA. In this work, it was also shown that Lon-dependent RovA proteolysis is strongly dependent on temperature. In this context it seems to be very likely that degradation signals are normally buried within the active RovA. However, thermo-induced change of conformation results in loss of DNA-RovA complex formation (inactive form) and consequent exposure of the target sequence. The SoxS protein is part of the SoxRS (superoxide) regulon in *E. coli* and was shown to be protected from Lon degradation when bound to DNA (Shah & Wolf, 2006a). Comparison of the RovA structure in a non-DNA-bound conformation

with the DNA-bound conformation could help to get further information about the connection between DNA-binding and Lon-mediated proteolysis.
The RovA protein of the *Y. enterocolitica* O:8 strain 8081 was also demonstrated to be temperature-sensitive. In contrast, the RovA protein in all *Y. enterocolitica* O:3 strains is resistant to changes in temperature (F. Uliczka, unpublished data). Sequence alignments of this strain with the *Y. enterocolitica* 8081 strain revealed an exchange of a proline into a serine at position 98 (F. Uliczka, unpublished data). These findings were particularly surprising, being one amino acid responsible for a completely different expression pattern of *rovA* and *inv*. Introduction of the same amino acid exchange in RovA of Y. pseudotuberculosis lead to stabilization of the protein (F. Uliczka, unpublished data). However, CD spectroscopy revealed no structural differences of the mutated protein in comparison to the wild-type RovA (N. Quade, unpublished data). The close distance of amino acid 98 to the DNA-binding domain also raises the possibility that this residue is somehow involved in substrate recognition.

Although RovA degradation by the Lon and Clp protease is clearly dependent on temperature, alterations of the DNA-binding activity due to thermo-induced conformational changes seem to be much more critical for temperature-dependent *rovA* expression than changes of protein stability. Autoactivation of *rovA* transcription is strongly reduced at 37°C, even in a *lon/clp* mutant. These findings raise the question of why RovA is rapidly degraded under non-inducing conditions (37°C) even though the protein exists in its inactive conformation. Thermo-induced structural alterations led to a strong reduction in DNA-binding capacity but did not cause complete inactivation. Therefore, degradation of the regulatory protein might be necessary to prevent binding to other regulatory regions with higher affinity within the *Yersinia* genome. Furthermore, only minimal changes in a positively autoregulated system by binding of RovA to its own promoter at 37°C may lead to an inappropriate switch from low level to high level expression.

In this study, a significant impact of growth phase on RovA degradation has been demonstrated. Growth phase dependent proteolysis could be shown in *E. coli* for the Dps protein, which protects DNA under nutrient stress conditions. Under nutrient limiting conditions Dps is stable, while it is rapidly degraded by the ClpXP proteases

upon entering into exponential growth phase. Subsequently, the presence of stabilizing protein factors similar to the RpoS-targeting adapter protein RssB expressed in stationary growth phase has been postulated (Stephani *et al.*, 2003). Investigation of temperature and growth phase dependent synthesis of the Lon and ClpP proteases in *Y. pseudotuberculosis* revealed significantly higher levels of proteases at 37°C compared to 25°C, but no differences in exponential and stationary growth phase. Therefore, it is likely that increased Lon synthesis at 37°C also contributes to thermoregulated proteolysis. However, this does not explain the differences in RovA stability in exponential and stationary growth phase. Thus, one or more additional factor(s) was/were hypothesized to contribute to growth phase dependent RovA proteolysis. Such factors may either affect the enzymatic activity of the protease or the conformational state and accessibility of the RovA protein.

In vitro degradation experiments of RovA demonstrated a significantly slower proteolytic process compared to the *in vivo* situation, although the protease was still active. In contrast, rapid degradation of RovA was observed when a crude extract of an exponentially grown *lon* mutant was added to the system, supporting the hypothesis of an additional component necessary for Lon-mediated RovA degradation.

Lon- and Clp-mediated proteolysis is known to be influenced by adaptor proteins that bind to the protease and confer substrates, enhance recognition and modulate activity. In *E. coli* four small adapter proteins have already been identified. The adapter protein SspB is required for the ClpXP-mediated proteolysis of the transmembrane protein RseA (Flynn *et al.*, 2004), whereas proteolysis of the general stress response sigma factor RpoS is controlled by the phosphorylated form of the adapter protein RssB (Muffler *et al.*, 1996). The adaptor protein ClpS directly interacts with the ClpA protease and preferentially recognizes N-end rule substrates. Simultaneously, ClpS inhibits degradation of SsrA tagged proteins (Kirstein *et al.*, 2009). Chaperones and proteases that create recognition sites or help to expose the signals to the Lon protease are also known to serve as accessory factors for efficient proteolysis. The molecular chaperones DnaK, DnaJ and GrpE together with ClpB were shown to promote formation of protease-substrate complexes by keeping the substrate in a soluble form, thus rendering them more accessible to the proteases (Huang *et al.*, 2001).

Furthermore, activity of the Lon protease was shown to be positively or negatively influenced by small cellular effector molecules like polyphosphate, ions and metabo-

lites. Kuroda and co-workers demonstrated that binding of inorganic polyphosphate to the Lon protease stimulates degradation of ribosomal proteins and prevents complex-formation of Lon with DNA (Kuroda *et al.*, 2001). In *Agrobacterium tumefaciens*, the transcriptional regulator TraR involved in quorum sensing, changes its conformation to a protease-resistant form upon binding of its effector molecule N-3-oxooctanoyl-HSL (Zhu & Winans, 2001). Moreover, the zinc-responsive transcriptional regulator ZntR of *E. coli* is stabilized by binding to zinc and DNA (Pruteanu *et al.*, 2007).

Allosteric control of DNA-binding by MarR-type regulators is known to be modulated by small effector molecules (Wilkinson & Grove, 2006). So far, no such molecule has been found for RovA but it is very likely that RovA also undergoes ligand-induced conformational changes which could influence its growth phase-dependent degradation.

4.1.3 Small aromatic compounds are able to stabilize RovA

Experiments to identify an accessory factor controlling growth phase dependent RovA proteolysis suggested a small, heat stable, pH sensitive, non-proteinaceous molecule that is produced in the stationary growth phase under nutrient rich conditions. Stationary phase media supernatants from *Yersinia*, *E. coli* and *Salmonella* were able to prevent RovA degradation by the Lon protease in exponentially grown cells. This indicates that the molecule is released by several Enterobacteriaceae into the medium and can quickly be taken up by the bacterial cell. Therefore a direct uptake mechanism by membrane transporters or recognition by a two-component system is likely. Moreover, the small RNA binding protein CsrA and the cAMP receptor protein Crp were found to be involved in factor production or control of factor activity, indicating that the unknown factor could be a metabolic intermediate involved in carbohydrate or amino acid metabolism.

Many members of the MarR family of transcriptional regulators are known to bind diverse anionic lipophilic, usually phenolic effector molecules. Structural data of the multiple antibiotic resistance regulator MarR from *E. coli* and the multidrug resistance operon antirepressor MexR of *Pseudomonas aeruginosa* revealed a mechanism of ligand-binding in which DNA- and effector-binding domains overlap (Wilkinson & Grove, 2006). Ligand binding to a regulator induces conformational changes and was

shown to either attenuate or increase the DNA-binding capacity of the protein (Egland & Harwood, 1999, Providenti *et al.*, 2001). The crystal structure of MarR was solved bound to one of its putative effector molecule salicylate but also similar compounds such as benzoate, 2,3-dihydroxybenzoate, 2,3-dinitrophenol, menadion and plumbagin were shown to modulate MarR activity *in vivo* and *in vitro* (Cohen *et al*., 1993, Martin & Rosner, 1995, Alekshun & Levy, 1999a, Alekshun & Levy, 1999b, Chubiz & Rao, 2010). Some MarR homologues are involved in the regulation of aromatic compound synthesis and were found to directly associate with the phenolic molecule that they regulate. CbaR from *Comamonas testosteroni* for example binds to 3-chlorobenzoate or protocatechuate that antagonize DNA-binding, whereas binding to 3-hydroxybenzoate seems to increase the DNA-binding affinity (Providenti *et al*., 2001). Recently, Perera and Grove (Perera & Grove, 2010) have shown that uric acid is a ligand for the MarR-type virulence regulator PecS of *Erwinia* which was previously described for the MarR homologue HucR from *Deionococcus radiodurans* (Wilkinson & Grove, 2004). Whether one of those compounds is a natural ligand for the different MarR-like regulators is still unknown but it is likely that this class of regulators is able to bind a variety of structurally related molecules.

It was postulated that RovA contains a ligand-binding pocket located in the vicinity of its DNA-binding domain. Crystallization of the protein with salicylate confirmed this hypothesis. The salicylate bound structure was similar to the DNA bound structure, indicating that ligand binding does not largely alter the structure of the protein (N. Quade, unpublished data).

In vitro binding experiments in this study demonstrated that small aromatic compounds similar to salicylate were able to stabilize the RovA structure. Those compounds are mostly involved in amino acid metabolism, nucleic acid metabolism, ubiquinone biosynthesis or tetrahydrofolate biosynthesis. Cinnamate, an intermediate of tryptophane biosynthesis was found to bind to RovA with the highest affinity of all tested molecules, whereas uric acid and phenylalanine were not able to bind to the protein. Additionally, it was found, that the position of the hydroxyl group on the benzyl ring is strongly influencing ligand binding, whereas the presence of an amine group lead to a complete loss of RovA binding affinity.

Taken together, *in vitro* as well as *in vivo* data demonstrated that small aromatic molecules with similar structure are able to stabilize the RovA protein and prevent degradation in exponential growth phase (C. Mendonca, unpublished data). Slight de-

gradation of RovA at 37°C in stationary growth phase indicates that ligand binding alone does not prevent RovA proteolysis completely. DNA-binding at 25°C in exponential growth phase in the absence of an effector molecule was shown to reduce but not abolish RovA degradation. However, the combination of DNA and effector binding at 25°C in stationary growth phase lead to complete stabilization of the protein. A similar mechanism was shown for the zinc-responsive transcriptional regulator ZntR of *E. coli* that is involved in the regulation of zinc homeostasis. Binding of ZntR to DNA protects the protein from proteolysis due to overlap of the DNA-binding domain and the protease recognition site. Additionally, the presence of zinc further increased ZntR stability and completely prevents degradation by the Lon and ClpP protease (Pruteanu *et al*., 2007). Crystallization of RovA bound to both, DNA and the native ligand molecule could help to understand the structural alterations that lead to increased protein stability. Moreover, Band Shift analysis could give a first idea if ligand binding increases or attenuates the DNA-binding capacity of the protein.

Whether one of the identified aromatic compounds acts as a natural ligand for RovA remains unclear. Relatively high K_d values in a millimolar range suggest that high concentrations of ligand are required to stabilize RovA. Binding studies with MarR and other MarR homologues show similar or much lower dissociation constants. The affinity of salicylate to MarR for example was found to be at a K_d of 0.9 mM, whereas 2,3-dihydroxybenzoate bound to MarR with a K_d of 0.5 mM (Chubiz & Rao, 2010). However, for both molecules it is not clearly demonstrated that they are natural ligands for MarR. In contrast binding of the natural ligand uric acid to HucR of *D. radiodurans* displayed a K_d value of 12 µM (Wilkinson & Grove, 2005). Considering that salicylate for example is toxic to bacteria at higher concentrations, it might be possible that binding of the natural ligand occurs at much lower concentrations than observed in this study. The toxicity of the other identified molecules *in vivo* still needs to be investigated.

Furthermore, a genetic screening of *Yersinia* mutants that possess a modified RovA stability could help to find the metabolic pathway involved in factor production. So far, *E. coli* mutants impaired in 2,3-dihydroxybenzoate biosynthesis, anthranilate synthesis and TolC dependent excretion, kindly provided by Chubiz and Rao (Chubiz & Rao, 2010), are under investigation (C. Mendonca, unpublished data).

Furthermore, analytical analysis of stationary phase supernatant could help to identify the native ligand of RovA. So far, HPLC-MS and GC-MS analysis of stationary

phase supernatant was not successful. In general concentration in the total amount of compounds in the nutrient rich medium necessary to allow the synthesis of the RovA-stabilizing factor was too high to identify a certain type of molecule. Thus, strong concentration of the medium and treatment with different solvents and resins before analysis could circumvent this problem.

4.1.4 Model of temperature and growth phase dependent regulation of *rovA* expression

In this study it was shown that RovA is a thermo sensing protein which responses to environmental temperatures directly by alteration of its conformation controlling its DNA-binding affinity and susceptibility to degradation by the Lon and ClpP proteases. Furthermore, small aromatic effector molecules were found to stabilize RovA in the stationary growth phase. Based on this data, a model shown in Figure 4-1 for temperature and growth phase dependent *rovA* regulation can be proposed.

At moderate temperatures in the exponential growth phase RovA exists in its active conformation and binds cooperatively to its own promoter region. Interaction with the DNA stimulates transcription by direct activation of the RNAP and anti-repression of H-NS mediated silencing (Tran *et al*., 2005). Those RovA molecules not bound to DNA are not or only slightly degraded by Lon and to a small extent by the ClpP protease. This leads to high amounts of RovA protein in the cell. A temperature shift to 37°C leads to an immediate conformational change. This results in a more inactive form of the RovA protein characterized by a lower cooperative DNA-binding capacity to the *inv* and *rovA* promoter regions. Additionally, loss of DNA-binding renders the amino acids in the proximity of the DNA-binding domain more accessible for the proteases leading to increased degradation. In stationary growth phase a lipophilic aromatic effector molecule binds to RovA at both temperatures. Moreover, DNA-binding in combination with effector binding blocks the protease recognition side subsequently leading to RovA stabilization. At 25°C in stationary growth phase RovA proteolysis is completely prevented, since the protein is in its active conformation, the ligand is bound and RovA is bound to DNA. This represents conditions outside the host. At 37°C RovA is present in its inactive conformation and exhibits a reduced DNA-binding functions which leads to lower RovA levels in the cell due to the positive au-

toregulatory circuit. Complete proteolysis does not occur since factor binding stabilizes RovA.

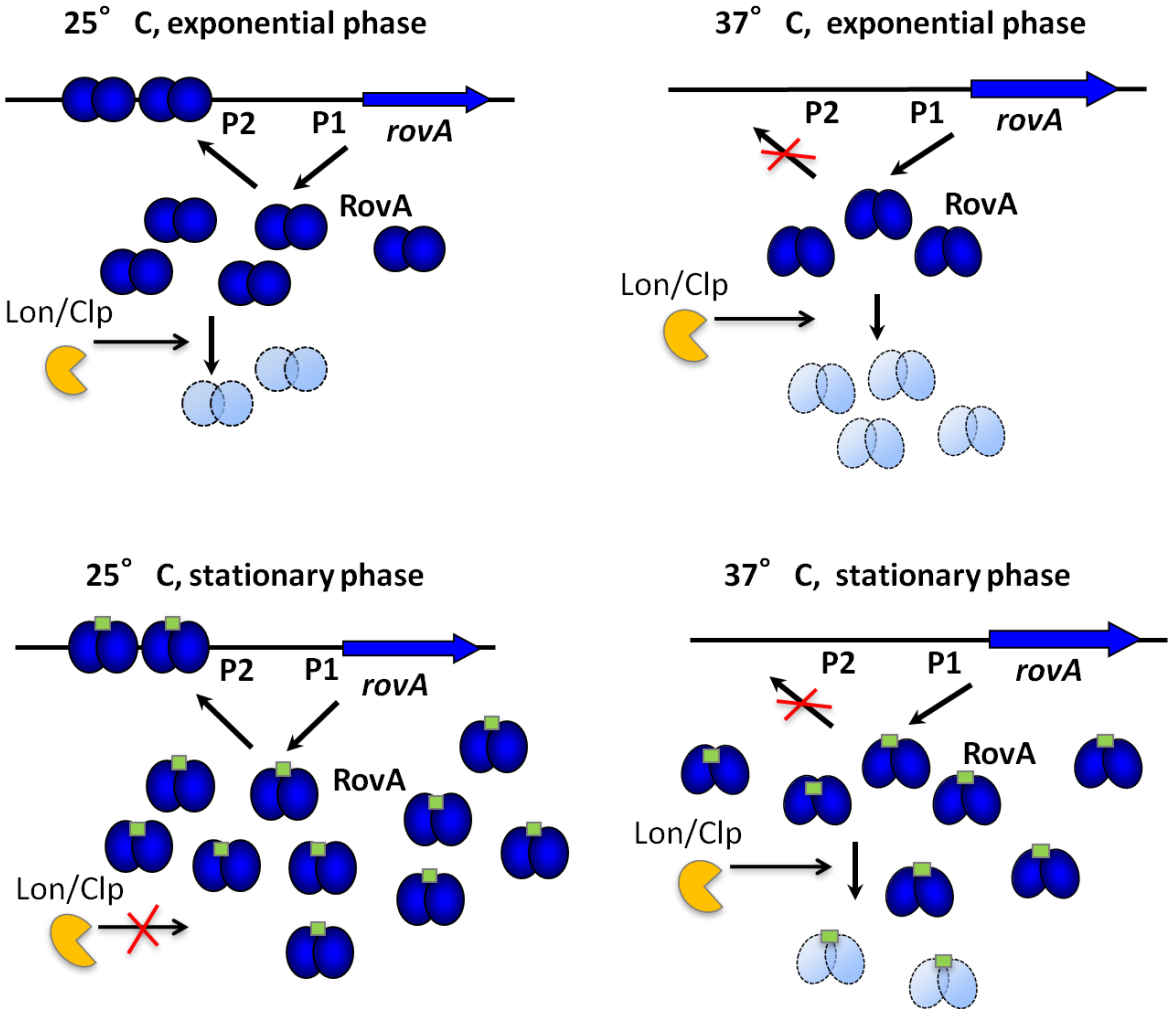


Figure 4-1. Model of temperature and growth phase dependent regulation of RovA. At 25°C in exponential growth phase RovA exists in its active form and induces its own expression. The protein is slightly degraded by the Lon and Clp proteases. At 37°C RovA undergoes a temperature induced conformational change reducing its DNA-binding affinity. Subsequently, the amino acid residues in the vicinity of the DNA binding domain are more accessible for the proteases and RovA is rapidly degraded. In the stationary growth phase an unknown factor most likely a small aromatic molecule stabilizes the RovA protein and locks the regulator in its active DNA-binding conformation which leads to stabilization of RovA at 25°C. At 37°C RovA exists in its inactive form and DNA-binding is reduced. During stationary growth phase the RovA-stabilizing factor prevents proteolysis by the Lon and ClpP proteases.

4.1.5 Impact of temperature and growth phase dependent RovA regulation on the RovA regulatory network and *Yersinia* virulence

Successful survival of a pathogen inside and outside a host requires a tight control of virulence gene expression. For *Yersinia*e, a very complex network has been established to switch between the expression of early and late virulence genes. Data obtained in this study, increased the understanding about the temperature-dependent expression of early virulence genes and gave a deeper insight into the RovA regulatory network presented in chapter 7 (Figure 4-2).

Outside the host environment *Yersiniae* show a free-living or food-associated life style. Under these conditions temperature is usually lower than 30°C and nutrients are generally scarce, similar to stationary growth phase. At moderate temperatures YmoA is expressed stable and represses the expression of LcrF/VirF, the key regulator of the late virulence genes. In addition, YmoA induces synthesis of CsrC leading to sequestration of the RNA binding protein CsrA. The sequestration of CsrA indirectly leads to repression of the *rovM* transcription leading to reduced RovM levels. As a consequence RovM does not interact with the binding site upstream of the *rovA* promoter P2 and does not block autoactivation at P1. RovA exists in its active, DNA-binding conformation and is stabilized by an effector molecule which completely prevents degradation by the Lon and Clp proteases. Thus, the protein exists in sufficient amounts to induce its own transcription, leading to the expression of early virulence genes.

During the ongoing infection, *Yersiniae* are confronted with higher temperatures and life conditions in the gut more comparable to exponential growth phase. Here, YmoA is rapidly degraded by the Lon and ClpP proteases and is no longer able to repress VirF/LcrF expression. This leads to induction of late stage virulence factor synthesis. Moreover, CsrC synthesis is repressed and CsrA is free to activate *rovM* expression resulting in repression of *rovA* transcription. Furthermore, RovA becomes inactive at 37°C and is rapidly degraded by Lon and Clp proteases. Low amounts of inactive RovA in the cell are not able to repress H-NS silencing, leading to complete repression of RovA-mediated early virulence genes.

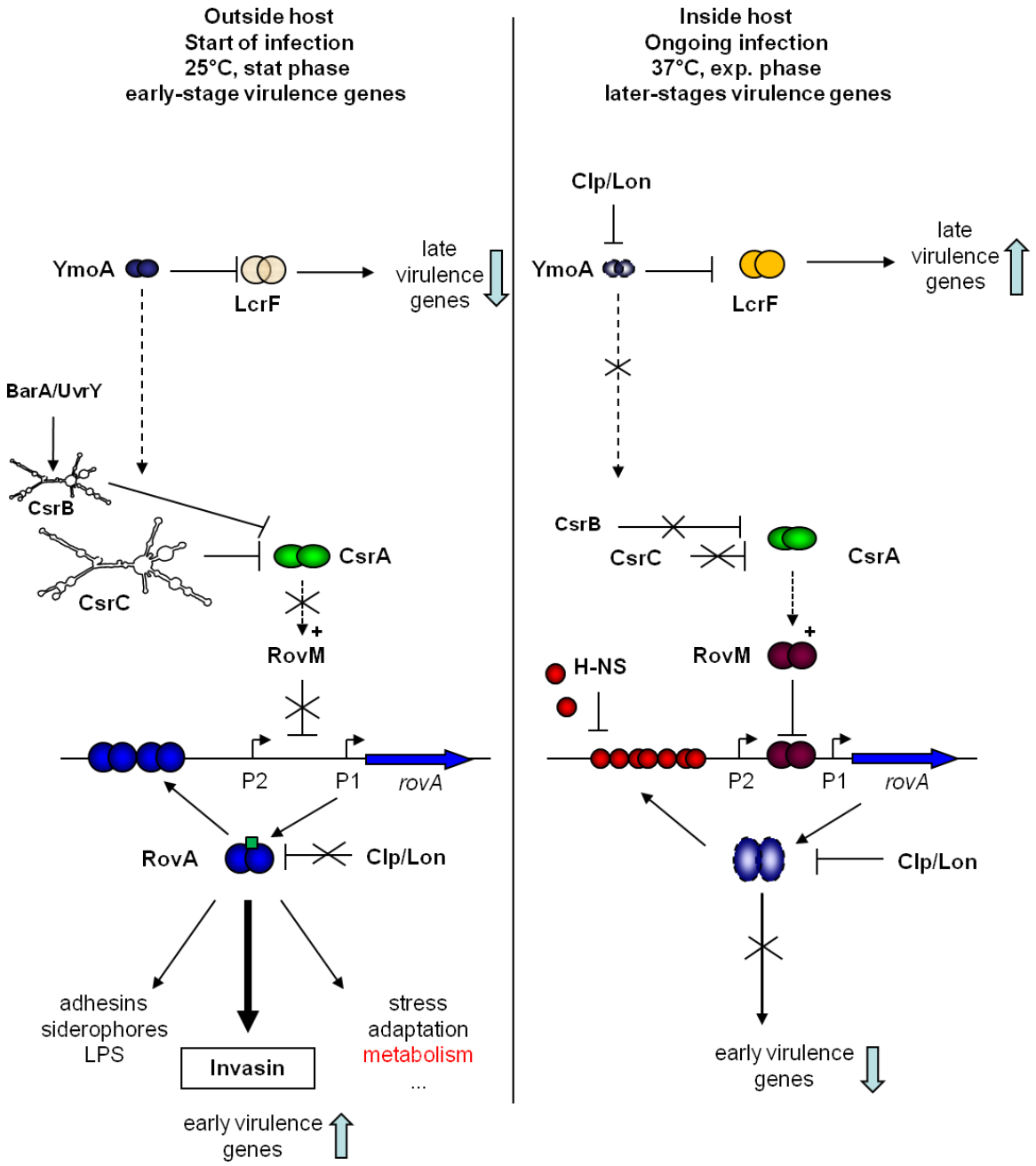


Figure 4-2. Extended model of RovA regulation. Compared to the model presented in Figure 1-7 the focus in this model lies in the temperature and growth phase dependent regulation of RovA. At moderate temperatures in stationary growth phase YmoA is expressed and leads to expression of the early virulence gene via the Csr-RovM-RovA pathway. RovA is present in its active, effector-bound form and is therefore protected from proteolysis. This results in activation of its own transcription as well as transcription of early virulence factors. Inside the host environment at 37°C, under conditions resembling exponential growth phase, YmoA and RovA are degraded by Lon and Clp proteases leading to expression of *rovM* and late virulence genes. *rovM* expression in combination with H-NS silencing leads to repression of RovA-induced expression of early virulence factors.

According to this model, temperature seems to play the most important role in coordination of virulence gene expression in *Yersiniae* on transcriptional as well as on post-transcriptional level. To guarantee the precise switch of the genetic program from early to late virulence gene expression the pathogen has evolved a sophisticated control system. Besides the direct temperature dependent post-translational activation or inactivation of the RovA protein, temperature dependent *rovA* expression is indirectly regulated by YmoA. At 37°C YmoA is degraded by Lon and Clp proteases and leads to increased RovM levels in the cell, blocking *rovA* expression. Both systems alone seem to be sufficient to inactivate *rovA* expression, but in case of failure of one system the other is still capable to down-regulate RovA expression, implicating repression of early virulence genes in the later stages of infection. These regulatory concepts together provide a new and interesting strategy to modulate and rapidly adjust a pathogen's virulence gene expression program during the course of infection.

4.2 Heterogeneous *rovA* expression and bistability

As already discussed previously, the adaptation to changing environmental conditions, such as entry from an e.g. food-associated lifestyle into the host environment, is regulated via complex gene regulatory networks, involving transcriptional, translational and post-translational mechanisms. However, in case of harsh and rapidly changing environmental conditions that exert a sudden selective pressure on a bacterial population, the time needed to respond within a gene regulatory network may be disadvantageous for population survival. Therefore, phenotypic heterogeneity, characterized by variable gene expression patterns within an isogenic population, is a sophisticated strategy to pre-adapt to sudden changes in the environment and thus, ensure survival of a bacterial subpopulation.

In this study it was demonstrated that *rovA* is expressed heterogeneously. Investigation of a *rovA*-*gfp*LVA fusion in *Y. pseudotuberculosis* YPIII revealed two distinct populations at 32°C during exponential growth. One population expressed *rovA* whereas the other one did not. Moreover, at higher temperatures no *rovA* expression was observed, whereas at 30°C almost all cells were expressing the *rovA* gene. Based on these results it was suggested that heterogeneous *rovA* expression origi-

nates from the temperature and growth phase-dependent post-translational regulation mechanism described in this study.

The existence of phenotypic heterogeneity in one population is well characterized and can arise from a variety of genetic and epigenetic mechanisms such as genetic rearrangements, mutations or DNA modifications. In a genetically homogenic culture of *Salmonella enterica* serovar Typhimurium for example, two populations expressing different types of flagella were found. This observation has been explained as the pathogen's attempt to avoid recognition by the immune system. The expression of different flagellin genes is mediated by a DNA recombinase that changes the orientation of a DNA-fragment including the flagellin gene cluster promoter (Zieg *et al.*, 1977, Simon *et al.*, 1980).

Moreover, stationary phase bacterial populations are often heterogeneous. The different populations are generated through mutations that change bacterial properties, leading for example to growth advantages of the newly arising population. The new population will suppress growth of the previous population, resulting in a mixed population (Finkel & Kolter, 1999).

A different mechanism for generating phenotypic heterogeneity is bistability, a situation in which two alternative gene expression states coexist within one population and reversibly switch between each other (Smits *et al.*, 2006). Bistable switches are generated by stochastic fluctuations in synthesis and stability of a master regulatory gene product, e.g. a transcriptional regulator. These fluctuations are referred to as noise. Noise-driven overshoot of a gene's expression threshold can lead to a completely new expression pattern and the population bifurcates into two subpopulations. A prerequisite for the formation of subpopulations is a positive or double negative feedback loop within the regulatory network of a transcriptional regulator. Moreover, the regulator needs to response to itself in a nonlinear manner. A nonlinear response may occur for instance by cooperative binding to the promoter region or degradation of the transcriptional regulator. This makes a bistable system extremely sensitive to regulator concentrations. When the amount of a transcriptional regulator in some cells exceeds its specific threshold, positive autoregulation leads to even higher accumulation of the protein. Thus, gene expression is altered, creating two distinct populations. These populations will coexist until the regulator concentration declines

below the threshold level, leading to transition from one stable steady state into the other (Dubnau & Losick, 2006, Ferrell, 2002, Mitrophanov & Groisman, 2008).

Bistable behaviours have been observed in a variety of cellular processes. For instance, competence and sporulation of *Bacillus subtilis* display characteristics of bistability, resulting in phenotypic heterogeneity. Competence is part of a physiological state, the so-called K-state, and occurs in 10% to 20% of stationary phase cells. The master regulator of competence in *B. subtilis* is the transcriptional regulator ComK. Its activity is regulated by proteolytic degradation, quorum sensing and transcriptional control (Smits *et al.*, 2005). Moreover, ComK is autoregulated. ComK autoregulation was shown to be responsible for competence bistability. When the concentration of ComK exceeds a certain threshold in the cell, a positive autoregulatory loop is activated, resulting in activation of the competence state (Maamar & Dubnau, 2005, Dubnau & Losick, 2006). Spore formation is an adaptive response to nutrient-limiting conditions. Within a starving culture, some cells initiate sporulation while other cells do not. The master regulator for sporulation is the response regulator Spo0A which is activated via a phosphorelay that is subject to three positive feedback loops. Veening and co-workers demonstrated that bistability of sporulation is mediated by the autostimulatory activation of Spo0A and can be modulated by quorum sensing-regulated phosphorelay phosphatases (Veening *et al.*, 2005). Furthermore, bistable expression of *spo0A* was suggested to influence biofilm formation (Chai *et al.*, 2008). In contrast, Chastanet and co-workers postulated that sporulation heterogeneity is solely generated by noise and that none of the known feedback loops serve as a bistable switch (Chastanet *et al.*, 2010). Recently, several other bistable systems have been discovered, including CsgD-mediated biofilm regulation in *Salmonella enterica* serovar Typhimurium (Grantcharova *et al*., 2010), ToxT-dependent expression of cholera toxin and in *Vibrio cholerae* (Nielsen *et al.*, 2010) and BexR-induced virulence gene expression in *Pseudomonas aeruginosa* (Turner *et al.*, 2009).

Mechanisms resembling bistable switches were also discovered in eukaryotes. The best-documented example is the maturation of *Xenopus laevis* oocytes. This process is controlled by the Mos-Mek-MAPK kinase cascade, embedded in a multicomponent positive feedback loop. The activator of the cascade is the steroid hormone proges-

terone, which determines the start of oocyte maturation in a concentration-dependent manner (Ferrell, 1999, Nebreda & Ferby, 2000).
Also for RovA a bistable behavior was postulated. Mathematical modelling of *rovA* expression revealed that positive autoregulation in combination with temperature-dependent proteolysis can lead to the appearance of bistability, indicated by two steady states for one temperature (Klein, 2010). Moreover, cooperative binding of RovA to its own promoter region was shown to facilitate switch-like transitions between the two stable steady states. High RovA levels at moderate temperatures (25°C), which are necessary for efficient colonization of host tissues, represent the first steady state. The second steady state is found at higher temperatures (37°C), resembling the conditions inside the host, where *rovA* expression is turned off. The temperatures at which the two stable steady states occur were shown to be determined by temperature-dependent RovA degradation and temperature-dependent promoter binding (Klein, 2010). In fact, two subpopulations representing the “ON” and “OFF” state of *rovA* expression could be detected during growth at 32°C.

In contrast to the postulated RovA system, in most of the bistable systems the switching-stimulus is mediated by activation or inactivation of the transcriptional regulator through phosphorylation or dephosphorylation (Maamar & Dubnau, 2005, Veening *et al*., 2005, Dubnau & Losick, 2006). A proteolysis-dependent switching mechanism was shown for cell chaining in *B. subtilis* (Chai *et al.*, 2010a). Cell chaining is controlled by autolysins, whose genes are controlled by a heterodimeric complex consisting of the proteins SlrR and SinI. Complex formation of SlrR and SinI is governed by a double negative feedback loop. This loop is a bistable switch existing in a $SlrR^{Low}$ state in which autolysin genes are switched on (no chaining) and an $SlrR^{High}$ state in which autolysin genes are repressed by the SinR-SlrR complex (chaining) (Chai *et al.*, 2010b). Cells in the $SlrR^{Low}$ state are converted into the $SlrR^{High}$ state by the antirepressor SinI that inhibits SinR and leads to SlrR expression. For the reverse transition a mechanism was found which involves autocleavage and ClpC-dependent proteolysis of SlrR (Chai *et al*., 2010a). Thereby, SlrR levels are a consequence of the relative states of synthesis and degradation in the early and late stages of biofilm formation (Chai *et al*., 2010a).
Another example for proteolysis-mediated heterogeneity is the already mentioned ComK system. Expression of *comK* is positively autoregulated and ComK binds to its

own promoter as a tetramer (Hamoen *et al.*, 1998). Such multimerization of the transcriptional regulator leads to a nonlinear response to the *comK* promoter. Similar to the *rovA* system, additional proteins bind to the *comK* promoter, activating or repressing *comK* transcription (Maamar & Dubnau, 2005). DegU increases promoter affinity of ComK whereas AbrB, CodY and Rok act as repressors on the *comK* promoter, thereby influencing the ComK threshold in the cell (Maamar & Dubnau, 2005). This tight transcriptional control was suggested to determine the threshold of ComK that is necessary to stimulate the positive feedback loop (Smits *et al.*, 2005). The basal level of ComK on the other hand determines the fraction of cells that reach this threshold and consequently develop competence. The basal ComK level is controlled by the adapter protein MecA (Turgay *et al.*, 1998). MecA binds to ComK at the onset of stationary growth phase and targets it for degradation by the ClpP and ClpC proteases. With increasing cell density, quorum sensing induces synthesis of ComS. ComS binds MecA and prevents targeting of ComK for degradation (Solomon *et al.*, 1995). However the bistable expression of *comK* was shown to be predominantly mediated by autoregulation (Smits *et al*., 2005, Maamar & Dubnau, 2005).
The molecular mechanism of bistability in *Bacillus* competence is similar to that of heterogeneous *rovA* expression. Besides positive autoregulation and nonlinearity both systems are regulated by numerous factors influencing gene transcription and, thus, threshold levels of regulator in the cell. However, in contrast to ComK, fine-tuning of RovA levels occurs by a Lon- and ClpP-dependent degradation mechanism triggered by temperature-induced conformational changes and the DNA-binding ability of the protein. Up to now, a similar mechanism to induce population heterogeneity has not been demonstrated.

The switching-ability of populations is thought to have evolved as an adaptation to fluctuating environmental conditions (Dubnau & Losick, 2006). The benefit of coexisting phenotypes is that although one part of the population is killed due to environmental stress, the other part remains viable and can give rise to a new population. Due to the fact that the selected properties are not genetically determined, the surviving population is able to generate all the phenotypes that were present in the initial population (Smits *et al*., 2006). Variable phenotypes in pathogenic bacteria might support evasion from the host immune response.

The benefit of heterogeneous *rovA* expression for *Yersinia* is still unclear. Heterogeneity is mainly observed at 32°C *in vitro*. This temperature is not encountered by the bacteria inside the mammalian host. It is possible that bistable behavior contributes to the adjustment to temperature fluctuations prior to host infection. However, it is more likely that other factors in the host environment, influencing the stability of the RovA protein, might shift the formation of subpopulations to higher temperatures. To clarify the impact of culture heterogeneity under physiological conditions, *rovA* expression needs to be monitored *in vivo*.

5 Outlook

Regulation of the global transcriptional regulator RovA occurs via a complex regulatory network, involving a variety of different regulatory elements. In this study, a post-transcriptional mechanism for temperature- and growth phase-dependent RovA regulation was discovered. Temperature-induced conformational changes of the protein lead to alterations in its DNA-binding affinity, making the protein more accessible to the Lon and ClpP proteases. However, the mechanism of temperature sensing by RovA is still unclear. Interestingly, the RovA homologue SlyA of *E. coli* which is 77 % identical to RovA is not able to sense temperature. Therefore, exchange of selected amino acids in RovA against the corresponding amino acids in SlyA could help to identify the temperature sensitive portion of the protein.

RovA degradation experiments revealed that an unknown accessory factor is able to stabilize RovA in stationary growth phase. Although it was shown that small aromatic molecules, similar to salicylate, are able to stabilize RovA, the native ligand of the protein has not yet been identified. The setup of a genetic screening system could be a reasonable approach to identify relevant genes for factor synthesis. Moreover, concentration of stationary phase supernatant and adsorption to different resins and solvents prior to HPLC-MS or GC-MS analysis could help to directly identify the unknown component in the growth medium.

Furthermore, heterogeneous gene expression of *rovA* was suggested to be mediated by temperature-dependent degradation and altered DNA-binding affinity due to conformational changes of the protein. To get a deeper insight into the molecular mechanism leading to heterogeneity of *rovA* expression, further experiments should be done, addressing the role of positive and negative feedback loops of *rovA* expression, protein degradation and involvement of other known RovA regulators. To link these results to the proposed bistability model, single cell analysis of *Y. pseudotuberculosis* cells harboring a *rovA-gfp* promoter fusion need to be performed demonstrating temperature-dependent switching between the “ON” and “OFF” state of *rovA* expression.

6 Summary

Temperature is one of the most crucial signals sensed by pathogenic bacteria to adapt their gene expression program to changing environmental conditions inside and outside the host organism. In *Yersinia pseudotuberculosis* expression of early virulence genes is regulated by the global transcriptional regulator RovA. *rovA* expression was found to be maximally induced at 25°C in stationary growth phase, whereas minimal *rovA* expression was observed at 37°C during exponential growth. Although a variety of regulators has been identified within the RovA regulatory network, none of them was found to be directly responsible for temperature- and growth phase-dependent *rovA* expression. In this work a post-transcriptional mechanism for RovA regulation in response to these environmental factors was demonstrated.

In this study it was shown that RovA acts as an intrinsic protein thermometer that controls its DNA-binding ability by temperature induced structural alterations. Cooperative binding to its own promoter region was strongly impaired at 37°C, indicating that temperature control of *rovA* transcription is mainly based on autoregulation. In addition, thermo-induced unfolding and, accordingly, reduced DNA binding under non-inducing conditions was found to increase the susceptibility of the protein to degradation by the Lon and ClpP proteases. An amino acid motif in the proximity of the DNA-binding region appears to contain the recognition signal necessary for proteolysis.

Furthermore, it was shown that a secreted factor is responsible for growth phase-dependent regulation of RovA synthesis. This factor prevents RovA degradation in stationary growth phase. ThermoFluor analysis revealed that salicylate and structural related metabolites are able to interact with and stabilize RovA, indicating that the secreted factor might be a small aromatic molecule with a salicylate-like structure. The affinity for the ligand is depending on the hydroxyl group position on the aromatic ring. The production of the RovA-stabilizing factor occurs under nutrient rich conditions in stationary growth phase and was demonstrated to be controlled by the sRNA binding protein CsrA and the cAMP receptor protein Crp.

Furthermore, it was shown that the *rovA* gene is heterogeneously expressed. Two distinct populations could be observed at 32°C during exponential growth, representing the “ON” and “OFF” state of *rovA* expression. At higher temperatures no *rovA*

expression was observed, whereas at lower temperatures the protein was synthesized in all cells. Based on the known *rovA* regulatory mechanisms it is suggested that heterogeneous *rovA* expression originates from bistability mediated by positive autoregulation, cooperative DNA-binding and temperature-dependent degradation of RovA.

Taken together, the temperature and growth phase dependent RovA regulation and the resulting heterogeneous gene expression both constitute a new example of how a microbial pathogen is able to rapidly adjust its virulence gene expression program to changing environmental conditions during the course of an infection

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8 Supplements

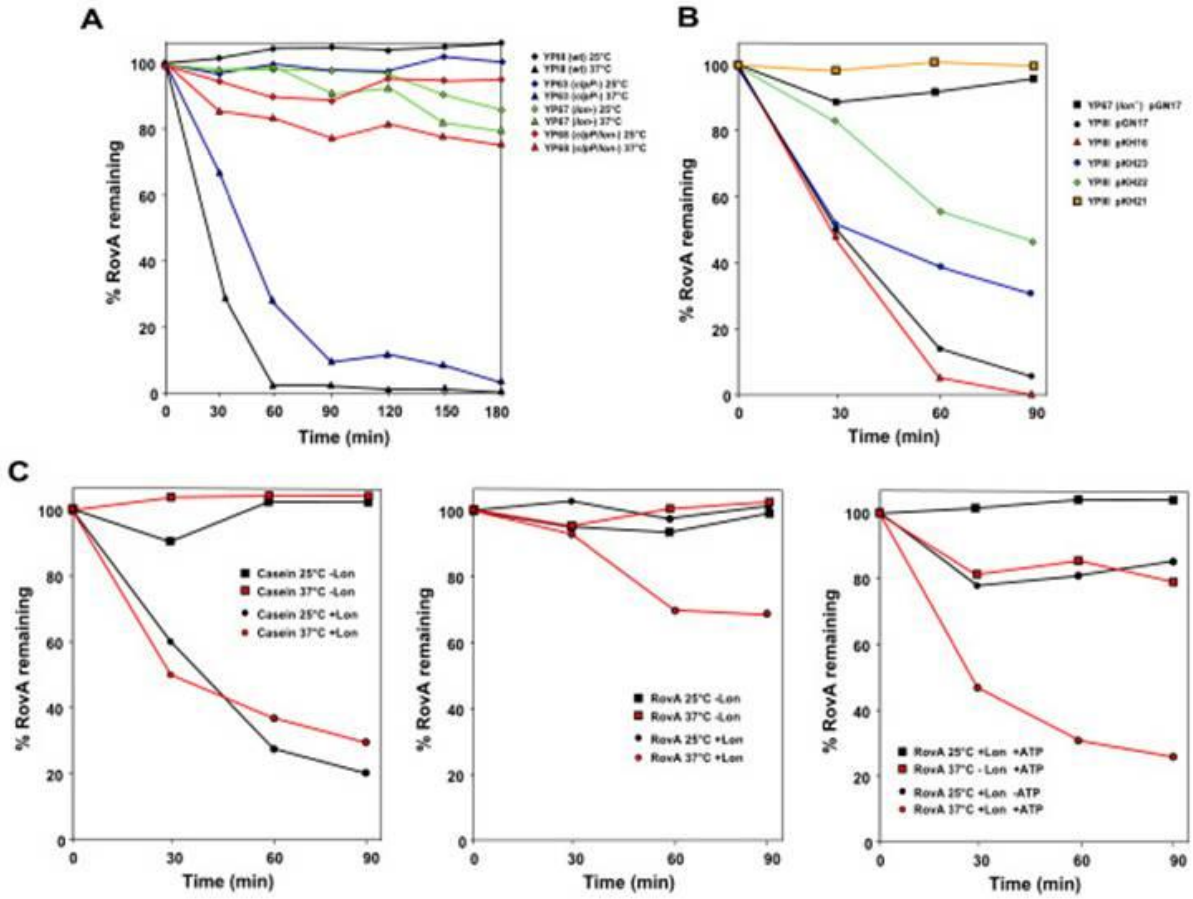

Figure S1. Quantification of RovA degradation. (A) *In vivo* degradation of RovA in YPIII (wt), YP63 (Δ*clpP*), YP67 (Δ*lon*) and YP68 (Δ*clpP/lon*) at 25°C and 37°C. **(B)** *In vivo* degradation of RovA-LacZ hybrids in (wt), YP63 (Δ*clpP*), YP67 (Δ*lon*) and YP68 (Δ*clpP/lon*) 37°C. **(C)** *In vitro* degradation of casein (left panel) and RovA (middle panel) at 25°C and 37°C with or without Lon. *In vitro* degradation of RovA (right panel) in the presence of whole cell extracts with or without Lon and ATP.

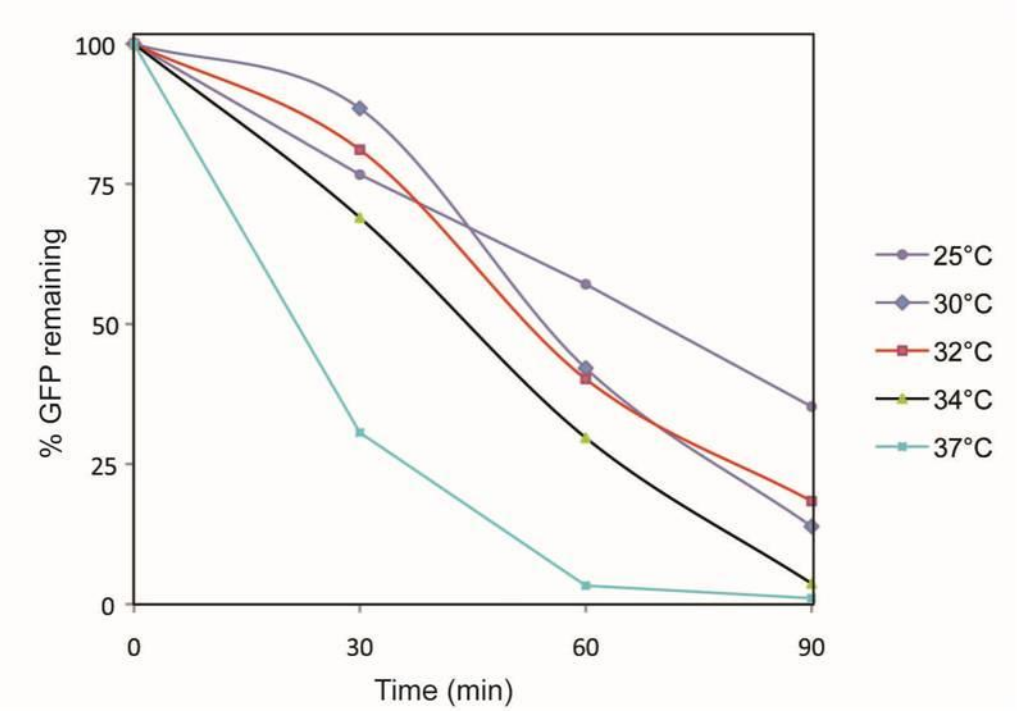

Figure S2. Quantification of GFP degradation. *In vivo* degradation of GFP in YPIII pKH70 (*rovA-gfp*) at 25°C, 30°C, 32°C, 34°C and 37°C.

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